

THE B-SPECIFIC DNA MODIFICATION ENZYME
OF
ESCHERICHIA COLI B

(L'ENZYME DE MODIFICATION D'ESCHERICHIA COLI B)

THESE

présentée à la Faculté des sciences
de l'Université de Genève
pour obtenir le grade de docteur ès sciences biologiques

par
URS KUEHNLEIN
de
Küsnacht (ZH)

Thèse No 1532

Genève
Imprimerie de l'Ecole de Physique

1970

UNIVERSITE DE GENEVE
Laboratoire de Biophysique

FACULTE DES SCIENCES
Professeur W. Arber

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La faculté des sciences, sur le préavis du professeur
W. ARBER, directeur de thèse, et du professeur P-F. SPAHR,
autorise l'impression de la présente thèse, sans exprimer
d'opinion sur les propositions qui y sont énoncées.

Genève, le 20 juillet 1970
Thèse No 1532

Le vice-doyen
Jean MULLER



SUMMARY

An enzymatic activity, having the properties expected of a B-specific host-controlled modification enzyme, has been purified from an extract of Escherichia coli strain B. This activity renders the unmodified replicative form of phage fd DNA resistant to B-specific restriction and is only present in strains carrying intact genes for type B modification.

In phosphate buffer, the enzyme acts optimally at pH 6.0 and is dependant upon a single cofactor, S-adenosylmethionine.

The modification is a specific methylation of certain adenine moieties of the DNA. The methylated adenine derivative which is formed has been identified as 6-methylaminopurine.

The unmodified replicative form of a mutant of phage fd, which has lost the sites which are sensitive to the action of the B-restriction enzyme, does not accept methyl groups in the modification reaction. This result indicates that the two enzymes, one for restriction, one for modification, both recognize the same specific sites on the DNA.

RESUME

Un extrait de la souche B de Escherichia coli a été purifié: cet extrait présente l'activité enzymatique attendue d'un enzyme de modification de type B. La forme replicative non modifié de l'ADN du phage fd est rendue, par cet extrait, résistante à la restriction spécifique de la souche B. Seules les souches porteuses des gènes fonctionnels pour la modification de type B présentent cette activité.

Le pH optimum de l'enzyme est 6.0 en tampon phosphate et la réaction dépend d'un seul cofacteur, le S-adenosyl-methionine.

La modification est la methylation spécifique de certaines adénines de l'ADN; le produit formé est la 6-méthyl-aminopurine.

La forme replicative d'un mutant du phage fd qui a perdu les sites sensibles à l'action de restriction B, n'est plus méthylée par l'enzyme de modification. Ce résultat indique que les deux enzymes, l'enzyme de restriction et l'enzyme de modification, reconnaissent les mêmes sites sur l'ADN.

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I. INTRODUCTION

Several bacterial strains possess a specific "restriction" and "modification" system which enables them to distinguish between their own and foreign DNA. This distinction is based upon a chemical alteration which is imparted to the DNA by the host cell. DNA which lacks this modification is degraded after penetration into the cell, whereas modified DNA is resistant to the degradation mechanism.

The molecular mechanism of this phenomenon has been most thoroughly studied in strains of Escherichia coli, using bacteriophage λ as the principal tool of investigation (see Arber and Linn, 1969). The DNA of bacteriophage λ , as well as many other bacteriophages and bacterial DNA itself, are susceptible to host-specific modification and restriction. As an example, phage λ grown on E.coli K has a probability of only 10^{-4} to successfully infect E.coli strain B. The DNA of the phage penetrates into the cell, but since it lacks B-modification, it is degraded by the B-restriction system. However, the progeny of the phages which escape restriction, usually contains B-modified DNA and will be accepted in a second growth cycle on the same strain. Even though the modification of the DNA by the host cell is a chemical reaction which probably involves covalent bonding, modification is not heritable. Growth of B-modified phage λ on E.coli K, yields a progeny which is again susceptible to restriction in E.coli B.

Dussoix and Arber (1962) demonstrated that the complete breakdown of phage λ DNA in a restrictive host is preceded the appearance of large DNA fragments of approximately 5% or more of the length of the phage genome. It was primarily this observation which led to the idea, that the restriction process is initiated by a specific enzyme, probably an endonuclease,

which acts on defined sites on the DNA. This reaction renders the DNA accessible to further breakdown by the exonucleases that are present in the bacterial cell. The modification activity, on the other hand, could be an enzyme, which recognizes the same sites on the DNA and alters them in such a way, that they are no longer sensitive to the action of the restriction enzyme.

Among the strains of E.coli, six different host-specificity systems have been characterized. The genetic determinants for three of them (K, B and A) are located on the bacterial chromosome near the thr locus (Wood, 1966; Arber and Wauters-Willems, 1970). The genes for the other systems (N-3, P1 and 15) are carried by plasmids (Watanabe et al, 1964; Lederberg, 1957; Arber and Wauters-Willems, 1970). The different host-specificity systems are independent in the sense, that modification in a given system does not influence the degree of restriction in any other system. A plausible explanation of this observation is that the enzymes of different host-specificity systems recognize different sites on the substrate DNA.

Strong evidence for this model came from investigations of the B-restriction of phage fd. Two mutants could be isolated from this phage, a single-step mutant which has a reduced sensitivity towards B-restriction and a double-mutant which has no sensitivity towards B-restriction at all (Arber and Kühnlein, 1967). The mutants were interpreted to have a mutation in one, or both sites, respectively, which are recognized by the B-restriction enzyme. These results were underlined by studies of the restriction of phage λ in the A-specificity system. In this system, phage λ has one specificity site which can be lost by mutation (Arber and Linn, 1969). In both cases, the sensitivity towards other host-specificity systems is not affected.

Recently, the restriction enzymes have been isolated from extracts of E.coli K and E.coli B (Meselson and Yuan, 1968; Linn and Arber, 1968). The two enzymes are indeed endonucleases which are able to cleave specifically unmodified or inappropriately modified DNA. In the case of the K-restriction enzyme (endonuclease R·K) with λ -DNA as the substrate, the endproduct of the reaction are double-stranded DNA fragments with a length of approximately one-fifth of the λ genome. Similarly, the B-restriction enzyme (endonuclease R·B) cleaves the circular double-stranded replicative form of phage fd into two fragments, thus confirming the genetic results which predicted two B-specific sites per molecule. Both the K- and the B-restriction enzymes require double-stranded DNA as a substrate and depend on the presence of S-adenosylmethionine, ATP and Mg^{++} . Similar activities in vitro were demonstrated in strains lysogenic for phage P1 and strains carrying the episome N-3 (Meselson and Yuan, 1968; Takano et al, 1968). The P1-restriction enzyme has the same requirements as the enzymes mentioned above, whereas endonuclease R·N-3 seems to be independent of the presence of S-adenosylmethionine and ATP.

The first insight into the chemical nature of modification came from experiments carried out with a methionine auxotroph mutant of E.coli K. Deprivation of methionine during the growth cycle of phage λ on this strain, yielded a phage progeny which was incompletely modified (Arber, 1965 a). Since methionine is a precursor of S-adenosylmethionine, a cofactor of methyltransferases, the result suggested that modification is connected with DNA-methylation. More direct evidence came from measurements of the number of methylated bases of the DNA of phage fd. It was found that B-modified fd DNA contains two times more methyl groups than unmodified fd DNA. The methylated bases were identified as 6-methyl aminopurines (J.D. Smith, cited by Arber and Linn, 1969).

The aim of this thesis was to demonstrate B-modification in a cell-free system and to purify the responsible enzymatic activity. The substrate DNA used to detect B-modification activity was the double-stranded replicative form (RF) of phage fd DNA (see Marvin and Hohn, 1969). This DNA molecule is particularly well suited for this purpose. It is double-stranded and thus, since it had been shown that the restriction enzymes act only on double-stranded DNA, a more probable substrate for the modification enzyme than the single-stranded phage DNA itself. Furthermore, the molecule is circular and thus resistant towards the exonucleases present in crude bacterial extracts.

However, the main reason for the choice of fd RF as the substrate for the B-modification activity, was the ability of the RF to infect specially prepared cells, called spheroplasts (Benzinger, 1968). This infectivity assay allows one to test RF-molecules for B-modification. Spheroplasts prepared from strains carrying the genes for B-specificity restrict unmodified RF molecules, whereas B-modified RF-molecules are accepted and give rise to phage progeny. Thus, B-modification in vitro is expected to increase the infectivity of RF-molecules on B-spheroplasts.

This infectivity assay was used to identify B-modification activity in bacterial extracts. The activity was found only in strains carrying intact genes for B-modification. It depends on a single cofactor, S-adenosyl-methionine, and requires double-stranded DNA as a substrate. Further purification by chromatography on phosphocellulose and DEAE-cellulose, allowed the separation of the modification and the restriction activities. This observation indicates that the two reactions which govern host-specificity, restriction and modification, are catalyzed by separate enzymes. The high degree of specificity displayed by in vivo modification

seems to be preserved in the in vitro reaction, since in vitro B-modified fd RF is resistant towards B-restriction, but is still fully susceptible to the heterospecific P1-restriction.

Experiments with the purified enzyme fraction and radioactive S-adenosylmethionine revealed that the B-modification enzyme is a methylase which specifically methylates unmodified DNA. The replicative form of the fd DNA of the mutant, which has lost the sites which are recognized by the restriction enzyme, has simultaneously lost the capability to accept methylgroups. Thus, the two enzymes react with the same sites on the substrate DNA.

The B-modification enzyme is also able to methylate unmodified DNA extracted from bacteria and phage λ . In both cases, as well as with RF fd DNA, adenine bases are methylated to form 6-methylaminopurine.

II. MATERIALS AND METHODS

1. Materials

S-adenosylmethionine (SAM) was purchased as the iodic salt from Calbiochem. It was repurified by chromatography on Bio-Rex 70 (100-200 mesh). Batches of 20 mg of the salt were dissolved in 2 ml of 10^{-2} M potassium phosphate (pH 6.8). The solution was applied to a 1 ml column (Pasteur pipette) which had been equilibrated with the same buffer. The column was washed with 10^{-2} M potassium phosphate and then eluted with 3 N acetic acid. The flow rate was approximately 0.1 ml per min, and fractions of 1 ml were collected. The concentration of the SAM which eluted in a single fraction was determined by measuring the optical absorption at 256 m μ . The SAM was stored at -15°C and neutralized with NaOH immediately before use.

The tritiated S-adenosylmethionine (H^3 -methyl SAM), was a product from the Radiochemical Centre, Amersham. Two batches were used. According to the firm, Batch I had a specific activity of 4.2 C per mM and a radiochemical purity of 93% and Batch II a specific activity of 4.7 C per mM and a radiochemical purity of 96%. The radioactive concentration of the two batches was determined by comparison with the radioactivity of a standard sample of n-hexadecane-1, 2-T(n) (Radiochemical Centre, Amersham). The values were 1.46 mC per ml for Batch I and 0.85 mC per ml for Batch II. The SAM was delivered in diluted sulfuric acid (pH 2.5-3.5). It was stored frozen at -15°C and not neutralized when used.

Whatman P11 phosphocellulose was purchased from W. & R. Balston, Ltd., and DEAE-cellulose from Schleicher, Schuell & Co. Batches of 100 gr were suspended in 1 liter of distilled water and the fine grains were removed. Then the resin was suspended in 0.5 N NaOH and, after 20 min at room

temperature, washed on a Buchner funnel with distilled water until the filtrate was again neutral. Finally, the resin was suspended in 2 liters of 0.02 M potassium phosphate at the pH used for chromatography. The buffer was exchanged several times and the suspension stored at 4°C.

Sephadex G 100 was purchased from Pharmacia (Uppsala) and Bio-Rex 70 (100-200 mesh) from Calbiochem. Before being used, the two resins were equilibrated for several days at room temperature with the buffers used for chromatography. The Sephadex G 100 was incubated for 1 hour at 60°C before the column was poured, in order to prevent the formation of air bubbles.

Phosphorus-32 in the form of orthophosphate was purchased from the Radiochemical Centre (Amersham), adenosine triphosphate from Sigma Chemical Co. and endonuclease I from Worthington Biochemical Co. Streptomycin and lysozyme were purchased from Calbiochem. Streptomycin was dissolved in distilled water immediately before use. Lysozyme was dissolved in 10^{-2} M Tris-HCl (pH 8.0), at a concentration of 20 mg/ml, frozen and stored at -15°C. Thirty per cent bovine serum albumin, "Povite", for spheroplast assays was a product of the Biotest Serum Institute (Frankfurt). Bactotryptone, Bactoagar and yeast extract were from the Difco Laboratories (Detroit). Benzoylated, naphthoylated DEAE-cellulose (BNC) was a generous gift of Dr. T. Young.

All the other reagents used were purchased either from Fluka or from Merck AG.

2. Bacterial and phage strains

The following strains of E.coli were used : 991 (Arber, 1966), an $F^+ r_K^+ m_K^+$ strain; 993 (Arber, 1966), an

$F^+ r_K^- m_K^-$ strain; 2027 (Arber, 1966), an F^+ derivative of a K-B transduction hybrid $r_B^+ m_B^+ thr_B^+ leu_K^- met_K^-$; 1101 (Dürwald and Hoffman-Berling, 1968), an $F^+ K$ strain deficient in endonuclease I ($endo^-$); 519, an F^+ derivative of E.coli C (Bertani and Weigle, 1953); 707 (Wood, 1966), a $met^- gal^-$ derivative of strain Bc 251 ($r_B^+ m_B^+$); 834 (Wood, 1966), a nonrestricting and non-modifying ($r_B^- m_B^-$) mutant of 707; 837 (Wood, 1966), a nonrestricting ($r_B^- m_B^+$) mutant of 707; 2539, a partial diploid ($r_B^+ m_B^+ thr^- leu^+ / F' r_B^+ m_B^+ thr^+ leu^-$) derivative of Bc 251, prepared by S. Linn and C. Berg with the F-prime derived from KLFI (Low, 1968); 930, a Pl-lysogenic derivative of 803 (Wood, 1966) of phenotype $r_K^- m_K^- r_{Pl}^+ m_{Pl}^+$.

The strains 991 and 993 are hereafter referred to as "O"-strains and the strain 2027 as "B"-strain.

Phage fd is the strain isolated by Hoffman-Berling et al (1963). Phage fd 101 (Arber and Kühnlein, 1967) is a mutant that has lost one of the two B-restriction sites on the DNA and will be referred to by its genotype fd sB-1⁰sB-2. Phage fd 601 (Arber and Kühnlein, 1967) has lost both B-restriction sites by mutation and will be referred to as fd sB-1⁰sB-2⁰.

3. Growth of bacterial cultures, preparation of lysates of phage fd, and their titration

3.1. Media : (concentrations are given in weight per volume)

Tryptone broth : 1% Bactotryptone, 0.5% NaCl; pH 7.

Tryptone soft-agar : Tryptone broth supplemented with 0.7% Bactoagar.

Hershey soft-agar : 1% Bactotryptone, 0.6% Bactoagar, 0.8% NaCl, 0.24% dihydrated sodium citrate, 0.13% glucose; pH 7.

Hershey plates : 1.3% Bactotryptone, 1.2% Bactoagar, 0.8% NaCl, 0.24% dihydrated sodium citrate, 0.13% glucose; pH 7.

3.2. Procedures : Unless stated differently, bacteria were grown in tryptone broth. The titer of a culture was determined by counting in a Petroff-Hausser bacteria counter.

Phage fd lysates were either made in liquid media or on plates (plate stocks). In liquid media, the host bacteria were grown to a density of 10^7 cells per ml at 37° and infected with a multiplicity of at least one phage per bacterium. Incubation was allowed for about twelve hours and the bacteria were removed by centrifugation for 10 min at 4,000 g. A titer of 5×10^{11} phages per ml was usually obtained.

Plate stocks were usually used to adapt the phage to a bacterial strain with a different host-specificity system. An aliquot of 0.1 ml containing 10^8 phages was mixed with 0.3 ml of the bacterial strain which had been grown to a density of 4×10^8 cells per ml. Then, 2.5 ml tryptone soft-agar were added and the mixture was distributed on a tryptone plate. After twelve hours of incubation at 37°C , the top layer of the plate was collected into a tube and mixed thoroughly with 0.5 ml of chloroform. Centrifugation for 10 min at 4,000 g gave a clear supernatant which contained approximately 2×10^{12} phages per ml.

For the determination of the phage concentration in a lysate, 0.1 ml or less of an appropriate dilution were mixed with 0.3 ml of indicator bacteria (4×10^8 cells per ml) and 2.5 ml of Hershey soft-agar. The mixture was distributed on a Hershey plate and after twelve hours of incubation at 37°C the plaques which had appeared were counted.

4. Infectivity assay for the single-stranded and the replicative form of phage fd DNA (modified procedure of Benzinger, 1968).

4.1. Media : (concentrations are given in weight per volume)

"Tris concentrated" : 12.1% Tris-HCl, 2% KCl, 2% NH_4Cl , 0.66% Na_2HPO_4 , 0.35% Na_2SO_4 , 0.075 volumes of HCl conc.; pH 7.

Maaløe-Hanawalt medium (modified) : 1 liter contains 100 ml "Tris conc.", 2.5 ml 1 M MgCl_2 and 2.5 ml of 1% required amino acids. The amount of glucose added varied, and will be indicated for each experiment.

Hard top-agar : 1% Bactoagar, 1.5% Bactotryptone, 0.7% NaCl; pH 7.

Sucrose soft-agar : To 100 ml of melted hard top-agar were added : 55 ml of 1 M sucrose, 1.85 ml of 10% MgSO_4 . The mixture was cooled to 42°C in a waterbath, and 2.5 ml of 30% bovine serum albumin and 5 ml of a saturated culture of indicator bacteria were added. The sucrose soft-agar was prepared immediately before use.

EMB-glycerol plates : 1% Bactotryptone, 1.5% Bactoagar, 0.5% NaCl, 0.2% K_2HPO_4 , 0.1% yeast extract, 0.04% eosine, 0.0065% methylene blue; pH 7. After sterilization 50 ml of 20% glycerol were added per liter.

Assay mixture : 0.1 M sucrose, 0.525% bovine serum albumin, 0.125% MgSO_4 , 10^{-3}M Tris-HCl; pH 8.

4.2. Preparation of spheroplasts : One liter of Maaløe-Hanawalt medium, supplemented with 1.25 ml of 20% glucose, was inoculated with 1 ml of an overnight culture grown in tryptone broth, and incubated at 37°C with vigorous aeration. The limited amount of glucose present allowed the bacteria to grow to a density of 2 to 3×10^8 per ml. After twelve to sixteen hours of incubation, an additional 23.5 ml of 20% glucose were added and further growth was allowed for two hours. The cells, which reach a density of 4 to 5×10^8 per ml, were harvested by centrifugation (10 min at 4,000 g) and resuspended in 40 ml of buffer containing 0.2 M Tris-HCl (pH 8.0), 0.4 M sucrose and 0.25×10^{-3} mg/ml of the required amino acids. The cell suspension was incubated for 30 min at 37°C with aeration and then transferred into a storage bottle. The storage bottle was kept at room temperature and 0.8 ml of 30% bovine serum albumin, 0.5 ml of 0.2 M EDTA and 1.3 ml of a 2 mg/ml lysozyme solution were added. The action of the lysozyme was stopped after one and a half minutes by the addition of 1.5 ml of 10% MgSO_4 , and the bottle was placed into a waterbath at 37°C for an additional thirty minutes. Samples were examined in the microscope to ensure that the cells appeared as single spheres.

The spheroplast preparations were stored at 4°C . They could be used three hours after the preparation, and the cells stayed competent for two weeks. Maximal competence (defined as plaque forming ability upon infection with an RF fd DNA standard) was usually reached after two to three days of storage.

Strain 2027 was used to prepare B-spheroplasts and strains 991 and 993 for O-spheroplasts.

4.3. Infectivity assay : An appropriate RF aliquot (0.1 ml or less) was mixed in a wide tube (at least 15 mm in diameter) with 0.9 ml of assay mixture. The tube was placed in a waterbath at 37°C and 0.3 ml of the spheroplast preparation was added (without shaking the tube). After 8 min of incubation, the absorption process was stopped by the addition of 3 ml of sucrose soft-agar, and the mixture was poured on an EMB-glycerol plate. Plaques become visible after approximately twelve hours of incubation at 37°C.

The efficiency of plating varied with the age and the individual preparation of the spheroplasts. In the case where no host-specific restriction occurs, it usually ranged between 10^5 and 2×10^6 plaques per optical density unit (260 mμ) of RF. This value corresponds to between 10^{-8} and 2×10^{-7} plaques per RF molecule. Spheroplast preparations with a lower plating efficiency gave a yield of plaques which was not proportional to the RF concentration. The same effect occurred when the amount of RF in the absorption mixture exceeded 1.5×10^{-3} optical density units (260 mμ). Single-stranded fd DNA has an approximately 10-fold higher plating efficiency than double-stranded RF and caused no problems in the infectivity assay.

In every infectivity assay, three plates were made with varied amounts of RF in order to make sure that the saturation level of the spheroplasts was not reached.

5. Isolation of the replicative form of fd DNA (modified procedure of Komano and Sinsheimer, 1968)

Bacteria were grown in 8 liters of tryptone broth to a density of 3×10^8 cells per ml and infected with a multiplicity of 8 phages per bacterium. The aeration was reduced for 15 min in order to facilitate infection. After further incubation for 75 min with restored aeration, the infected cells were chilled,

harvested by centrifugation (10 min at 4,000 g), and washed twice with 2 l of 0.1 M NaCl, 0.05 M Tris-HCl (pH 8.0). The final pellet was resuspended in 160 ml of 0.5 M sucrose, 0.05 M Tris-HCl (pH 8.0). Eight ml of a 20 mg/ml lysozyme solution and 60 ml of 0.2 M EDTA were added and the mixture distributed immediately into screw cap polypropylene tubes of the Spinco No. 30 rotor (capacity of tubes 25 ml). After 10 min at room temperature, 1.5 ml of 10% Brij 58 (dissolved in 0.01 M Tris-HCl; pH 8.0) were added per tube. The tubes were kept at room temperature for an additional 10 min, and shaken gently from time to time. The solution became clear and highly viscous.

The cell debris and the high-molecular-weight DNA were removed by centrifugation in a Spinco No. 30 rotor for 20 min at 30,000 rpm (at a temperature below 5°C). The supernatant was extracted twice with phenol (saturated with 0.01 M Tris-HCl; pH 8.0) at room temperature and the two aqueous phases were pooled. Sodium acetate (2M; pH 5.4) was added to give a final concentration of 0.2 M and the nucleic acids were precipitated by adding 1.3 volumes of isopropanol and incubating overnight at -15°C.

The precipitate was pelleted by centrifugation for 1 hour at 14,000 g, the supernatant was discarded, and the isopropanol still present in the pellet was evaporated in an air stream. The precipitate was redissolved in 20 ml of 0.01 M NaCl, 0.02 M Tris-HCl (pH 8.0), and, for each optical density unit at 260 mμ, 2 μg of heat treated pancreatic RNase (8 min at 100°C) were added. The mixture was incubated for one hour at 37°C.

The low-molecular-weight material of the solution was removed by filtration on a Sephadex G 100 column (45 cm x 5 cm; length x inner diameter). The density of the sample was increased by the addition of 1 g of solid sucrose; and the solution was pumped between the eluant layer and the gel surface, using a parastaltic pump. The column was eluted with 0.3 M NaCl-TE (TE = buffer containing 10^{-3} M EDTA, 0.01 M Tris-HCl; pH 8.0).

The flow rate was 2 ml per minute, and fractions of 13 ml were collected. The first absorption peak at 260 m μ of the elution profile coincided with the infectivity measured in the spheroplast system (Figure 1). The fractions of this peak were pooled.

In order to separate the double-stranded RF from the single-stranded phage DNA, the pooled fractions of the Sephadex column were further chromatographed on a BNC column (benzoylated, naphthoylated DEAE-cellulose column). Before the application of the sample, the column (5 cm x 1 cm) was washed with 100 ml of 2 M NaCl-TE and 100 ml of 0.3 M NaCl-TE. Then the sample was applied, the column washed with supplementary 80 ml of 0.4 M NaCl-TE, and DNA was eluted with a 160 ml linear gradient of 0.4 M to 1 M NaCl-TE. The flow rate of the column was 0.3 ml per min, and fractions of 3 ml were collected. Under these conditions the double-stranded DNA eluted at a concentration of 0.54 M NaCl-TE; whereas the single-stranded DNA remained adsorbed to the resin.

The infectivity of the collected fractions was determined on O-spheroplasts and compared with the optical absorption at 260 m μ (Figure 2). The purest fractions were pooled, concentrated by dialysis against solid sucrose to a concentration of approximately 1 OD_{260 m μ} per ml, and then dialyzed against 0.01 M Tris-HCl (pH 8.0), containing 2×10^{-4} M EDTA.

As judged by the electron microscope observation (Figure 3), 70% to 80% of the DNA present in the purest fractions was RF. The contaminating DNA is probably of bacterial origin. The yield from an 8 l culture was between 0.2 and 0.3 mg of RF.

6. Isolation of P³²-labelled replicative form of phage fd DNA

6.1. Media : "TPG2A conc." : 12.1% Trizma base (Sigma Chemical Co.), 1.1% NH₄Cl, 8% KCl,

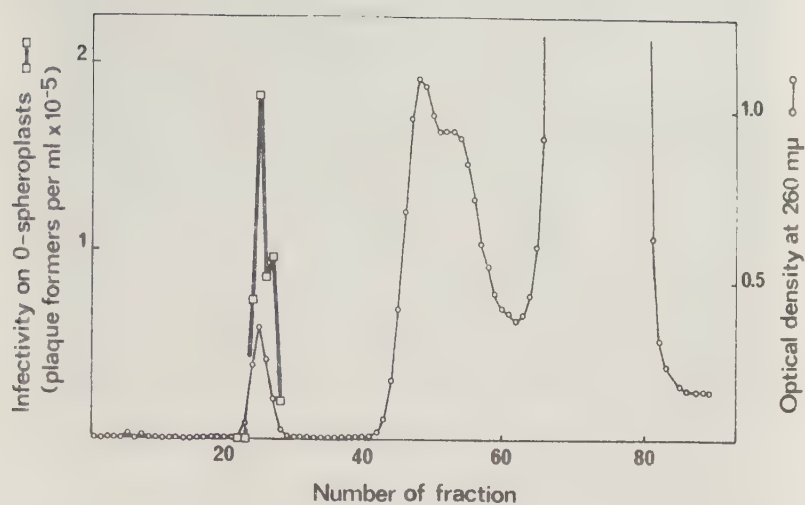


Fig. 1: Filtration of the RF-lysate on Sephadex G 100. The size of the column, the flow rate and the volumes of the fractions are given in the text. $\circ-\circ$, optical absorption at 260 m μ ; $\square-\square$, infectivity on O-spheroplasts.

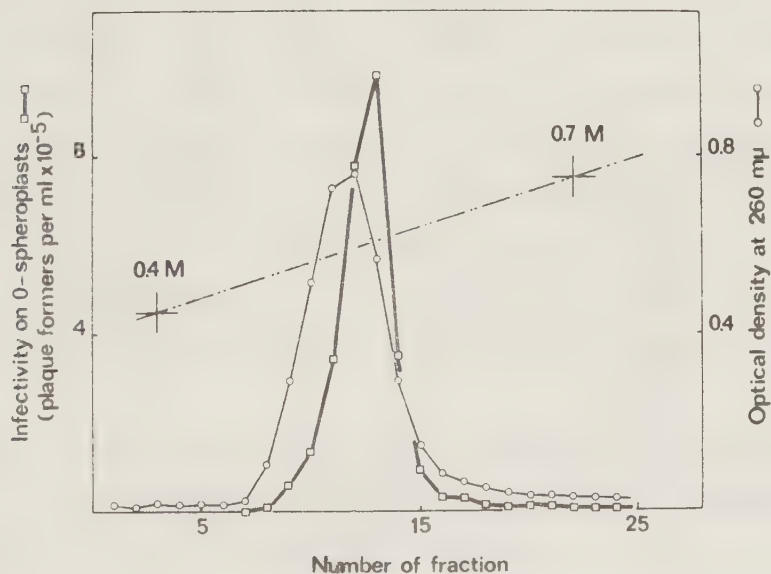


Fig. 2: Purification of the RF lysate by chromatography on BNC-cellulose. $\circ-\circ$, optical absorption at 260 m μ ; $\square-\square$, infectivity on O-spheroplasts. The details of the chromatography procedure are given in the text.

0.5% NaCl, 0.8% sodium pyruvate; pH adjusted to 7.4 with conc. HCl.

"TPG2A" medium : To 900 ml H_2O were added : 100 ml "TPG2A conc.", 1 ml of 1 M $MgCl_2$, 1 ml of 0.16 M anhydrous Na_2SO_4 and 2.5 ml of a 1% solution of the required amino acids. The solution was sterilized and supplemented with 2 ml of 0.5 M $CaCl_2$, 1 ml of 0.1 mg/ml $FeCl_2 \cdot 6 H_2O$, 20 ml of 20% glucose, and 10 mg of adenine. The concentration of KH_2PO_4 is varied, and will be indicated for each experiment (Lindquist and Sinsheimer. 1967).

6.2. Procedure : Bacteria were grown to a density of 2×10^8 cells per ml in one liter of "TPG2A" medium supplemented with $1.7 \times 10^{-3} M KH_2PO_4$. The cells were sedimented by centrifugation for 10 min at 4,000 g, and resuspended in 200 ml of the same medium without phosphate. The cell suspension was infected with phage at a multiplicity of 10. After 30 min at room temperature, 800 ml of "TPG2A" medium, which contained 1 mC of P^{32} (orthophosphate) and a total phosphate concentration of $1.7 \times 10^{-4} M$ (adjusted with KH_2PO_4), were added to the suspension. The culture was split in two aliquots and incubated in 2 l erlenmeyer flasks in a shaker bath for 75 min at $37^\circ C$.

The RF was isolated from the infected cells as described above, except that the volumes of the reagents used for lysis of the cells and the extraction of the DNA were reduced by a factor of eight.

During the isolation procedure, the radioactivity was measured in a Nuclear Chicago gas flow counter (Model D-47). Aliquots of 0.1 ml of an appropriate dilution in H_2O were dried on planchets and counted. The concentration of the

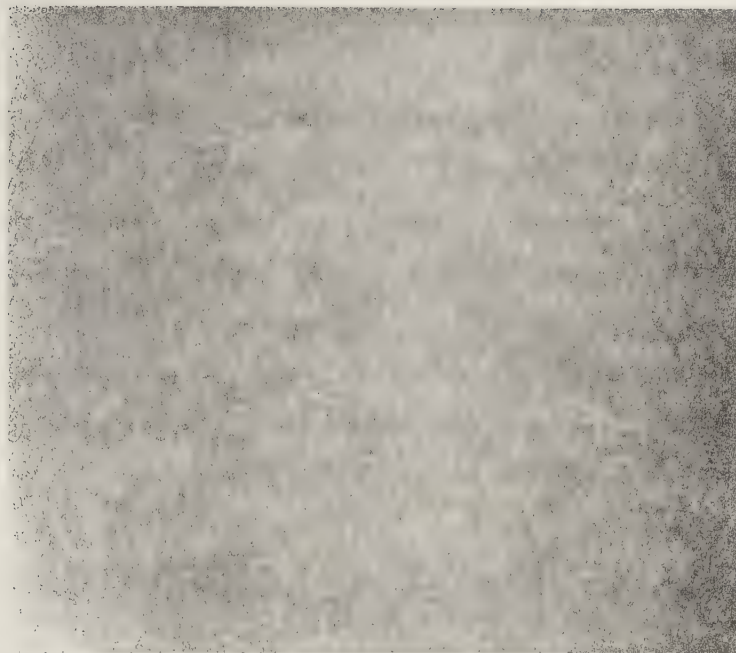


Fig. 3: Electron micrograph of the purified RF preparation. The sample was taken from fraction 13 of the BNC column (Fig. 2) and prepared according to the method of Kleinschmidt. (Courtesy of R. Bijlenga)

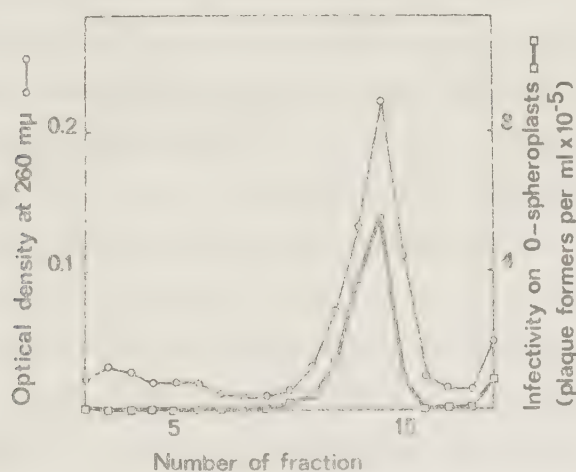


Fig. 4: Sedimentation profile of the purified single-stranded fd DNA. The sucrose gradient (5 ml) was run between 5% and 20% sucrose in 10⁻³ M EDTA, 0.01 M Tris-HCl (pH 8.0). Centrifugation was 3.5 hours at 10⁰ C in a SW 39 Spinco rotor at 36,000 rpm. The fractions were collected through a hole punched in the bottom of the tube. ○-○, optical density at 260 mμ; □-□, infectivity on O-spheroplasts.

purified RF was determined by comparing the infectivity on O-spheroplast with the infectivity of a standard RF sample.

In a first preparation with 1 mC of P^{32} per liter of culture medium, the uptake of label into the cells was 0.4 mC and the final RF preparation contained 0.34 μ C per mg RF (6.6×10^{-3} P^{32} -atoms per RF-molecule). In a second preparation, where 3.2 mC of P^{32} per liter were used, 1.3 mC were recovered in the washed cells. The amount of RF obtained was 0.47 μ C with a specific activity of 53 μ C per mg (2.3×10^{-2} P^{32} -atoms per RF-molecule).

7. Isolation of single-stranded phage fd DNA

A 2-liter lysate of phage fd was prepared in tryptone broth as described previously. The titer of the lysate was 5×10^{11} phages per ml. To the phage suspension, 160 g of polyethyleneglycol 6000 and 46 g of NaCl were added and dissolved, and the solution carefully mixed with 50 ml of 20% dextran sulfate. The solution was kept at 4°C overnight. Under these conditions the solution formed two phases and the phage precipitated at the interphase. The upper and the lower phases were removed and the precipitated phage resuspended in 15 ml of 0.01 M Tris-HCl (pH 8.0). The still turbid suspension was incubated with 0.25 mg of DNase I and 1.2 mg of RNase for 60 min at 37°C. The phage DNA was extracted twice with phenol (saturated with 0.01 M Tris-Cl, pH 8.0) at room temperature. The final aqueous phase was made 0.2 M sodium acetate (pH 5.5), and the nucleic acid precipitated by adding two volumes of isopropanol and incubating overnight at -15°C. The precipitate was collected by centrifugation for 1 hour at 14,000 g and redissolved in 20 ml of 0.3 M NaCl-TE. Then the DNA was purified on Sephadex G 100 as described for the isolation of RF. The DNA-containing fractions were applied to a 5 ml BNC column and washed with 1 M NaCl-TE. This buffer elutes RNA, double-stranded DNA and phage fd, whereas the single-stranded DNA

remains absorbed to the column resin. The single-stranded DNA was eluted with 1 M NaCl-TE containing 15 mg caffeine per ml. The first fraction of 5 ml contained 90% of the infectivity which eluted from the column. This was dialyzed extensively against 2×10^{-4} M EDTA, 0.01 M Tris-HCl (pH 8.0).

The total amount of single-stranded DNA obtained from the 2-liter lysate was 0.8 mg, which corresponds to 1.2×10^{14} molecules. A sample of the isolated phage DNA was analyzed on a sucrose gradient : the infectivity and the optical density sediment in a single peak (Figure 4).

8. Preparation of the "dialyzed ammonium sulfate fraction"

Bacteria were grown in one liter of tryptone broth to a density of 3×10^8 to 4×10^8 cells per ml, collected by centrifugation for 10 min at 4,000 g and resuspended in 6.5 ml (suspension buffer 5×10^{-4} M EDTA, 5×10^{-3} M 2-mercaptoethanol, 0.05 M Tris-Cl). The cell suspension was treated in an ultrasonic desintegrator until about 90% of the cells were disrupted, and the debris were removed by centrifugation for 10 min at 4,000 g. To the sonicate, a 1 M solution of $MgCl_2$ was added to give a final concentration of 0.035 M. The precipitate which had formed during incubation at $0^\circ C$ for 30 min was pelleted by centrifugation for 10 min at 20,000 g and discarded. The supernatant was diluted with suspension buffer to a concentration of 120 optical density units at 260 m μ . Then 0.72 volumes of a 5% streptomycin solution were added and, after 20 min at $0^\circ C$, the precipitate was again removed by centrifugation for 10 min at 20,000 g. The supernatant was made 60% saturated in ammonium sulfate by the addition of the dry salt, which had been previously grounded to a fine powder. After 30 min at $0^\circ C$, the precipitate which formed was collected by centrifugation for 10 min at 20,000 g, and redissolved in 2 ml of suspension buffer, and dialyzed twice versus 2 liters of 5×10^{-4} M EDTA, 5×10^{-3} M 2-mercaptoethanol, 0.01 M potassium phosphate (pH 6.8). The

turbidity which forms during the dialysis was removed by centrifugation for 10 min at 20,000 g and the extract was stored at 4°C.

The "dialyzed ammonium sulfate fraction" prepared in this way had, for all bacterial strains used an optical absorption ratio $A_{280\text{ m}\mu}/A_{260\text{ m}\mu}$ of approximately 1.0. Preparations with a ratio lower than 0.7 cannot be used because of the interference of the nucleic acids, still present in the extract, with the infectivity assay which was used to measure the modification and restriction activity of the extract.

It was observed that the extract loses approximately 50% of its enzymatic activity after 4 days of storage at 4°C. It was found later that the modification activity is more stable if the dialyzed ammonium sulfate fraction is stored in 50% glycerol at -15°C. The same is probably also true for the restriction activity.

9. Purification of the modification activity by chromatography on phospho-cellulose and DEAE-cellulose

All the buffers used during the purification contained 5×10^{-3} M 2-mercaptoethanol and 5×10^{-4} M EDTA, and all operations were carried out at 4°C.

The "dialyzed ammonium sulfate fraction" of the partial diploid strain 2539 was prepared from a 37 l culture. This fraction was subsequently applied to a phosphocellulose column (20 cm x 4 cm) which had been equilibrated with 0.01 M potassium phosphate (pH 6.8). The column was washed successively with 250 ml 0.01 M and 220 ml 0.05 M potassium phosphate (pH 6.8), and then eluted with a 2000 ml linear gradient of 0.05 M to 0.40 M potassium phosphate (pH 6.8). The flow rate of the column was approximately 0.9 ml per min, and fractions of 20 ml were

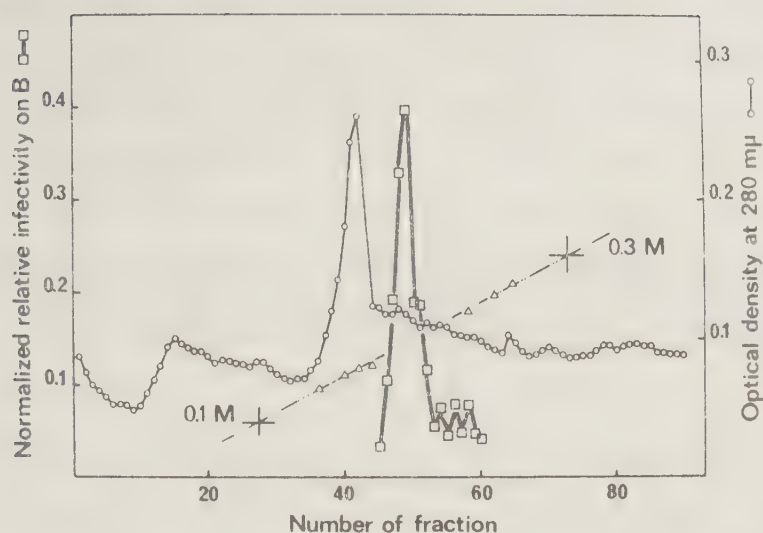


Fig. 5: Purification of the B-modification activity by chromatography on phosphocellulose. The "dialyzed ammonium sulfate fraction" obtained from the partial diploid strain was purified on a phosphocellulose column as described in the text. □-□, modification activity measured as the normalized relative infectivity on B of fd sB-1⁰sB-2 RF·0 after exposure to 40 μl of column eluate. ○-○, optical density at 280 mμ. The modification reaction was carried out at pH 6.0 using the standard conditions.

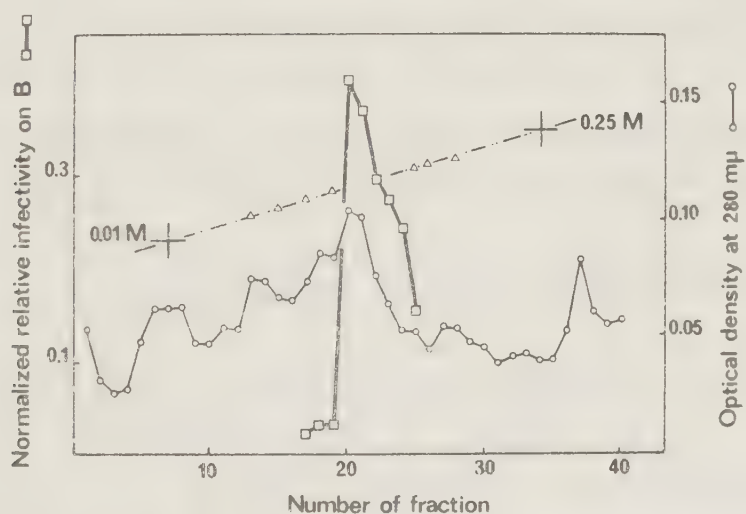


Fig. 6: Purification of the B-modification activity by chromatography on Whatman 40 DEAE-cellulose. The details of the chromatography procedure are described in the text. ○-○, optical density at 280 mμ. □-□, modification activity, measured as the normalized relative infectivity on B of fd sB-1⁰sB-2 RF·0 after exposure to 30 μl of the column fractions. The modification reaction was carried out using the standard conditions, except that the time of incubation was 50 min and the pH of the reaction mixture was 6.0

collected. The optical absorptions at 260 mμ and the modification activities of the fractions are represented in Figure 5. The modification activity eluted at a mean concentration of 0.18 M potassium phosphate and coincided with a little peak of the optical absorption profile, approximately ten fractions after the highest peak of the entire elution profile. This observation was made in three successive preparations and helped to localize the fractions to be tested for modification activity.

The three fractions containing the major modification activity were pooled, concentrated by dialysis against polyethyleneglycol 6000, and dialyzed overnight against 0.01 M potassium phosphate, pH 7.2. This material was applied to a Whatman 40 DEAE-cellulose column (6 cm x 1 cm) which had been equilibrated with the same buffer. The modification activity was eluted by means of a 150 ml linear gradient running from 0.01 M to 0.40 M potassium phosphate (pH 7.2). The flow rate of the column was 0.24 ml per minute, and fractions of three ml were collected. The major activity eluted in four adjacent fractions at a mean salt concentration of 0.15 M (Figure 6). These fractions were pooled, concentrated 10-fold by dialysis against PEG, dialyzed against 0.01 M potassium phosphate, diluted twofold with glycerol and stored at -15°C.

10. Detection of in vitro modification

10.1. Reaction using unlabelled S-adenosylmethionine as cofactor of the modification enzyme : Unless otherwise indicated, reaction mixtures (0.14 ml) contained 0.07 M potassium phosphate (pH 6.7), 1 mM 2-mercaptoethanol, 0.014 M EDTA, 130 μM S-adenosylmethionine (and consequently, 0.04 M sodium acetate arising from the acetic acid used to store the compound), and 0.02 optical density units (260 mμ) of RF. The amount of protein added is indicated in each assay. After incubation at 30°C for 30 min, the mixtures were chilled and assayed for infectivity on O-spheroplasts and B-spheroplasts.

B-specific modification of RF was detected by the increased relative infectivity on B-spheroplasts (infectivity of RF on B-spheroplasts divided by infectivity of RF on O-spheroplasts). The details of this assay system are discussed in Section III.

10.2. Reaction using H^3 -methyl S-adenosylmethionine as cofactor of the modification enzyme : Reaction mixtures contained 0.07 M potassium phosphate (pH 6.7), 1 mM 2-mercaptoethanol, and 7 mM EDTA. The amount of protein (only the purified enzyme fraction was used), RF and H^3 -methyl S-adenosylmethionine (SAM- H^3) are indicated in each assay. SAM- H^3 , stored in diluted sulfuric acid (pH 2.5 to 3.5), was added without being neutralized. This did not markedly change the pH of the reaction mixture. Incubations were carried out at 37°C. In addition to the RF to be tested, the reaction mixtures contained P^{32} -labelled fd sB-1⁰ sB-2⁰ RF·B which was either added before or after the incubation at 37°C. This RF does not accept methyl groups (see Section IV) and served to measure the recovery of RF after the treatment used to separate the DNA from the SAM- H^3 which had not undergone reaction.

The reaction was stopped by the addition of 75 μ l of 0.5 M sucrose, 0.7 M NaCl, 0.01 M glycine-NaOH (pH 9.5), and subsequent incubation at 65°C for 10 min. The sample (0.25 ml or less) was layered between the gel surface and the eluant layer of a 15 cm x 1 cm Sephadex G 100, column and eluted with a buffer containing 0.3 M NaCl, 10^{-3} M EDTA and 0.01 M Tris-HCl (pH 8.0). The flow rate was approximately 0.2 ml per min, and fractions of 1 ml were collected. Most of the RF was found in two fractions, separated from SAM- H^3 (Figure 7).

To the column fractions, 0.25 mg of carrier DNA were added (0.5 ml of a solution containing 0.5 mg of low molecular weight herring sperm DNA per ml and the DNA was precipitated by the addition of 4 ml 2N HCl at 0°C. The precipitate was

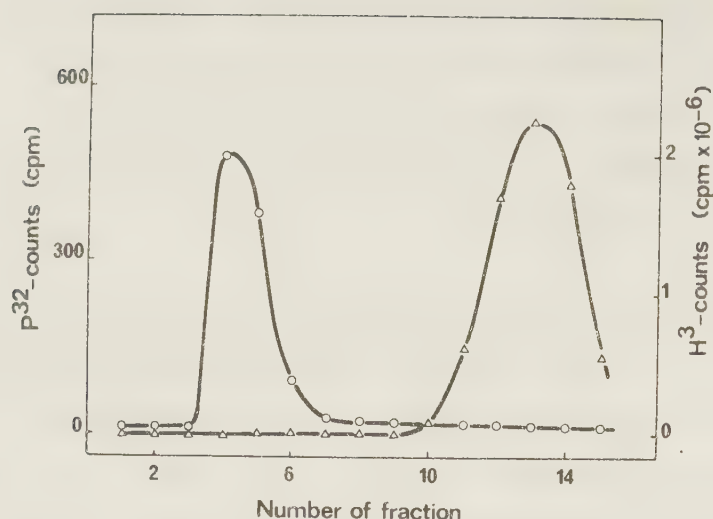


Fig. 7: Separation of RF- P^{32} and SAM- H^3 by filtration on Sephadex G 100. Samples of 200 μ l of the column fractions were dried on Whatman glass fiber filters (GF/C) and counted in a Beckman scintillation counter in the (H^3)-channel and the (P^{32} - H^3)-channel. $\circ$ - $\circ$, P^{32} -counts (cpm); Δ - Δ , H^3 -counts (cpm).

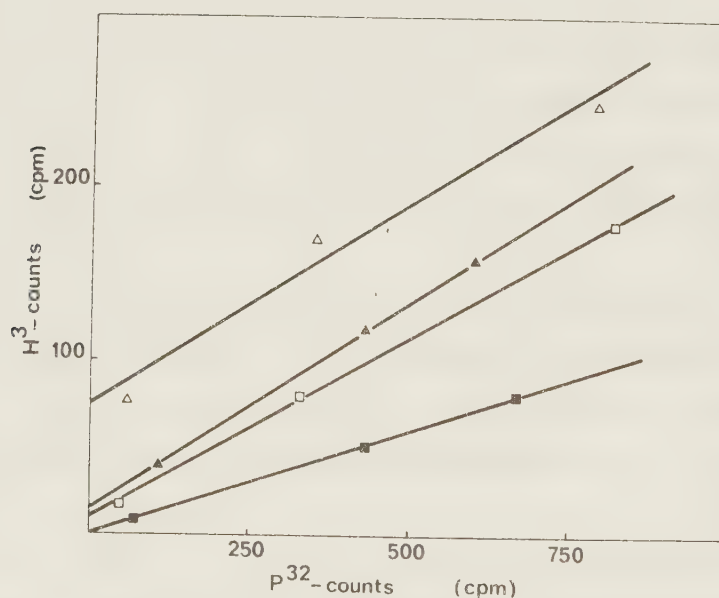


Fig. 8: Determination of the background radioactivity. For every fraction of the Sephadex column, the acid precipitable H^3 -counts were plotted versus the P^{32} -counts. The intercept with the ordinate gives the background. The examples represented here are taken from an experiment wherein the reaction velocity was measured as a function of the SAM- H^3 concentration (see Section IV). $\blacksquare$, 3.6 μ M SAM- H^3 ; $\square$, 27 μ M SAM- H^3 ; $\blacktriangle$, 36 μ M SAM- H^3 ; $\triangle$, 63 μ M SAM- H^3 . The background contributions increase with increasing SAM- H^3 concentration.

collected by filtration through Whatman glass fiber filters (type GF/C). The filters were washed successively with 2 x 4 ml and 2 x 20 ml 2 N HCl and 4 ml methylalcohol. They were dried and placed into scintillation vials. Then, 4 ml of toluene scintillation fluid was added to each, and radioactivity was counted in a Beckman liquid scintillation counter (Model LS 200 B) at a gain of 2.30 in the (H^3)-channel and the (P^{32} - H^3)-channel.

The H^3 -counts were first corrected for the counting background and the overlap of P^{32} -counts into the (H^3)-channel. A second correction was made for the background due to incomplete separation of SAM- H^3 and RF. This background was determined graphically by plotting the H^3 -counts versus the P^{32} -counts for each column fraction. A straight line was obtained, and the ordinate intercept was taken as the background value (Figure 8).

This background determination does not show contributions which are proportional to the RF concentration of the column fractions. Figure 9 shows the H^3 -counts obtained in a reaction, wherein the modification enzyme was replaced by a 50% glycerol solution, in dependence of RF concentration of the Sephadex column fractions. The RF-dependent background is 8 cpm per 10^{-3} mg RF. This background contribution was neglected in most of the experiments, since the RF concentration in any of the column fractions was less than 10^{-3} mg. In experiments wherein the reaction velocity was measured as a function of enzyme concentration or SAM- H^3 concentration, the RF-dependent background was taken into account; because the maximal RF concentration was between 2×10^{-3} and 3×10^{-3} mg per column fraction.

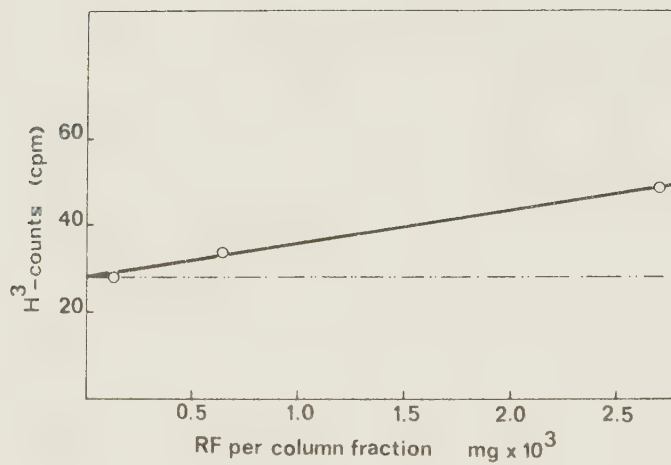


Fig. 9: Background radioactivity as a function of RF-concentration. The data were obtained from a control experiment for determining the velocity of the modification reaction as a function of enzyme concentration: in the control experiment the enzyme was replaced by 50% glycerol (see Section IV). In the figure, the acid precipitable H³-counts are plotted versus the RF concentration for each Sephadex column fraction. The DNA-dependent background is approximately 8 cpm per 10⁻³ mg DNA.

11. DNA hydrolysis and chromatography of the bases

After the modification reaction, the DNA was separated from SAM-H³ by filtration on a Sephadex G 100 column as described above. The fractions which contained the DNA were pooled, and 200 μ g of carrier DNA (low molecular weight herring sperm DNA) were added. The total DNA was precipitated with 5% TCA (30 min at 0°C), collected by centrifugation at 2,500 g for 10 min and redissolved in 0.5 ml of 0.2 M NaOH. After an additional precipitation with 10% TCA, the DNA redissolved in 0.5 ml of formic acid.

The formic acid hydrolysis of the DNA and the descending chromatography were then carried out as described in Methods of Enzymology (Vol. III, page 715). The chromatography separation of bases was made on Whatman No.1 chromatography paper in an NH₃-vapour phase with n-butanol-water (86:14, v/v) as the solvent. Markers of 6-methylaminopurine and 5-methylcytosine (4 OD₂₆₀ units) were chromatographed on the same sheet.

After approximately 30 hours, the chromatography was stopped and the bases located by UV light. The chromatogram was cut into strips, 2 cm wide and 1 cm in length (i.e. in the direction of the solvent migration). These were placed into vials, toluene scintillation liquid was added, and the radioactivity was counted in a liquid scintillation counter.

12. Other methods

Bacterial DNA was isolated according to the method of Marmur (1961), followed by a phenol extraction and filtration on a Sephadex G 100 column. The DNA of phage λ was a gift from D. Wauters-Willems. It had been isolated from purified phage by two successive cold phenol extractions.

Protein was determined by the method of Lowry et al (1951), DNA concentration by the method of Burton (see Methods in Enzymology, Vol. XII, page 163), phosphate concentration by the method of Chen et al (1956), and the ionic strength of potassium phosphate in gradient fractions by using a multi-vibrator conductivity meter.

III. ISOLATION OF THE MODIFICATION ENZYME AND ITS PROPERTIES, USING A TRANSFECTION ASSAY FOR THE DETECTION OF IN VITRO MODIFICATION

1. The assay system for B-specific modification

As we have pointed out previously, host-specific modification alters certain bases of the DNA and, as a biological consequence, renders the DNA resistant towards restriction. The chemical nature of this alteration is most probably a methylation of some of the adenine moieties of the DNA. Thus, in principle, modification activity in a cell-free extract could be detected by its ability to methylate specifically unmodified DNA. However, due to the rarity of specificity sites on DNA, this assay would require a radioactive methyl donor of a high specific activity. As a consequence, such an assay is rather expensive and the elimination of background radioactivity would cause technical difficulties.

We have therefore chosen a transfection assay for the monitoring of the B-modification activity in the steps of its purification. Such an assay had been used by Dussoix and Arber (1965), in order to demonstrate the association of modification with DNA. They showed that transfection of a restricting bacterial host with modified λ DNA is several orders of magnitude higher than transfection of the same host with unmodified DNA.

However, if a transfection assay is used for the detection of in vitro modification, λ DNA is not a suitable substrate. It is a linear molecule and thus sensitive to the presence of DNA polymerase and exonucleases in the cell-free extract. We therefore used the circular replicative form of phage fd DNA as a substrate for the detection of B-modification in vitro.

In the following paragraphs we will briefly describe the modification and restriction properties of phage fd and its

replicative form DNA and correlate the degree of modification of an RF sample with its efficiency of transfection of a B-restricting host.

1.1. Restriction and modification of bacteriophage fd :

○ Bacteriophage fd is small and filamentous, 50 Å wide and 2000 Å long, and contains a single-stranded circular DNA composed of about 6,600 nucleotides (see Marvin and Hohn, 1969). The phage is sensitive to two of the known host-specificity systems of E.coli, the B-system and the P1-system (Arber, 1966).

The degree of restriction of phage fd in the B-system is indicated in Table 1. It is measured as the relative infectivity on the B-restricting strain 2027, that is the infectivity on this strain divided by the infectivity on a standard non-restricting strain. In the case of phage fd, either a derivative of E.coli K (strain 991) or a restriction and modification deficient mutant (993) were used as standard strains. Hereafter they will be called O-strains, in order to express that phage fd is not restricted by these strains.

Phage fd grown on a O-strain (fd·O) has a relative infectivity on E.coli B of 7×10^{-4} , indicating that roughly one out of 1,000 DNA molecules which enter the cell escape restriction and give rise to phage progeny. On the other hand, phage grown on a B-strain are B-modified and have the same probability to successfully infect the restricting and the nonrestricting host cell. The restriction of phage fd by P1- lysogenic strains is less efficient than B-restriction. The relative infectivity of unmodified phage on this strain is 0.3. The two host-specificity systems are independent of each other; P1-modification does not influence the restriction of fd by E.coli B and vice versa.

The number of specificity sites on the DNA of phage fd which are sensitive to B-restriction are probably two. Indeed,

Phage	Relative infectivity on B
fd <u>sB-1</u> <u>sB-2</u> · 0	0.0007
fd <u>sB-1</u> <u>sB-2</u> · B	1
fd <u>sB-1</u> ⁰ <u>sB-2</u> · 0	0.032
fd <u>sB-1</u> ⁰ <u>sB-2</u> · B	1
fd <u>sB-1</u> ⁰ <u>sB-2</u> ⁰ · 0	1
fd <u>sB-1</u> ⁰ <u>sB-2</u> ⁰ · B	1

Table 1: B-specific modification and restriction of bacteriophage fd. The relative infectivity on B was determined by dividing the number of plaque formers on the B-strain 2027 by the number of plaque formers obtained on the O-strain 993. fd sB-1 sB-2 is the wild type phage. The symbols sB-1 and sB-2 indicate that this phage carries two B-specificity sites on its DNA, site number 1 and site number 2. The mutational loss of such a site is indicated by the superscript ⁰. The symbol after the dot designates the modification of the phage DNA. Thus fd·0 is fd grown on a O-strain and thus not modified, whereas fd·B is grown on a B-strain and thus B-modified.

mutants of phage fd could be isolated which have a relative infectivity on B of 3.2×10^{-2} as compared to 7×10^{-4} of the wild-type phage. This reduced level of restriction can be completely lost by a second mutation (Table 1). The interpretation of this finding is that the one-step mutant has lost one of the sites sensitive to cleavage by the B-restriction and that the double-mutant has lost the remaining site. Recently, this interpretation has been confirmed by genetic mapping of the B-specificity sites of phage fl, a phage closely related to phage fd (T. Boon, Personal communication). Hereafter, the two mutants will be referred to as fd sB-1⁰sB-2 and fd sB-1⁰sB-2⁰ respectively.

1.2. Restriction and modification of the replicative form of phage fd DNA : Upon penetration into the host cell, the single-stranded DNA of phage fd is converted into a double-stranded replicative form (RF). This replicative form undergoes further replication in the cell and a pool of 100 to 200 RF molecules is formed (see Marvin and Hohn, 1969). The RF molecules can be isolated and purified. They are able to infect specially prepared cells (spheroplasts) and to produce phage progeny (Benzinger, 1968). Both the isolation of RF and the infectivity assay are described in Materials and Methods.

The replicative form is subject to host-specific modification and restriction. B-spheroplasts degrade RF isolated from an fd infected O-strain, whereas modified RF (RF isolated from a B-strain) will be accepted and produce phage progeny. On the other hand, O-spheroplasts cannot distinguish between modified and unmodified RF.

The relative infectivity on B (infectivity on B divided by infectivity on O) which had been introduced as a measure for the degree of restriction of phage fd by strain B, has to be corrected in the spheroplast system for the variation of the competence of the two spheroplast preparations. The ratio of infectivity on B-spheroplasts to infectivity on O-spheroplasts was further divided by the relative infectivity on B of a

Type of RF	Infectivity on O (plaque formers per $OD_{260} \times 10^{-4}$)	Infectivity on B	Relative infectivity on B	Normalized relative infectivity on B
fd <u>SB-1</u> <u>SB-2</u> RF·B	34	490	14.4	= 1
fd <u>SB-1</u> <u>SB-2</u> RF·O	34	0.95	0.028	0.002
fd <u>SB-1</u> ⁰ <u>SB-2</u> RF·O	15	9.1	0.61	0.04

Table 2: Restriction of fd RF by B-spheroplasts. The infectivity assays with O- and with B-spheroplasts were carried out as indicated in Materials and Methods. The relative infectivity on B was determined by dividing the infectivity on B by the infectivity on O. These values were further divided by the relative infectivity of fd SB-1 SB-2 RF·B to give the normalized relative infectivity on B.

standard sample of B-modified RF (RF·B). Thus a normalized relative infectivity on B is defined, which is calculated in the following way :

$$\frac{\text{relative infectivity of RF sample}}{\text{relative infectivity of RF·B}}$$

The normalized relative infectivity on B of fd RF·B is by definition equal to one. The values for the unmodified RF of the wild-type phage (fd sB-1 sB-2 RF·O) and of the unmodified RF of the mutant fd sB^O-1 sB-2 (fd sB^O-1 sB-2 RF·O) are given in Table 2. They are the same as those obtained for the corresponding phages on untreated cells. This indicates that the conversion of bacteria into spheroplasts did not affect the restricting power of the cells.

1.3. Detection of B-modified RF : B-specific modification activity in a cell-free extract is expected to catalyze the reaction which converts RF·O into RF·B. Hence, the infectivity on B-spheroplasts of a sample of unmodified RF should increase after exposure to the modification activity in an in vitro reaction.

The number of sites which undergo modification during the reaction can be estimated from the normalized relative infectivity on B. In the case of the mutant fd sB^O-1 sB-2 (one restriction site per molecule), the frequency n of restriction sites which have been modified during the reaction is equal to the frequency of modified RF molecules. Therefore, the contribution to the normalized infectivity on B (R_B) of the modified RF molecules will be $n \times 1$ and the contribution of the remaining unmodified RF molecules will be $(1-n) \times 0.03$. Thus, the total normalized relative infectivity on B as a function of the frequency of modified sites is given by the following equation :

$$\begin{aligned} R_B &= (1-n) \cdot 0.03 + n \\ &= 0.03 + 0.97 n \end{aligned}$$

The basic assumption for the derivation of this equation is that stable partially modified intermediates showing reduced restriction do not exist.

In the case of the wild-type phage the calculation is more complicated, because the RF molecules carry two restriction sites. However, if we assume that the two sites are independent but have the same probabilities of being modified, and that the two possible intermediates have the same restriction as the the RF of the mutant fd sB-1⁰sB-2, we can derive the following equation :

$$R_B = 0.94 n^2 + 0.063 n + 0.001$$

For the isolation of the modification enzyme, the RF of the mutant fd sB-1⁰sB-2 was used as a substrate rather than the RF of the wild-type phage. The reason for this choice was the limited competence of the spheroplasts. The high level of restriction of the wild-type phage makes it necessary to infect B-spheroplasts with a high amount of RF in order to have a sufficient yield of plaques, with the consequence that the infectivity assay becomes unreliable.

2. Identification of the modification activity in the dialyzed ammoniumsulfate fraction

As we have pointed out, B-specific modification in vitro should convert fd RF 0 into fd RF·B and thus increase the infectivity of a sample of fd RF·0 on B-spheroplasts but not on O-spheroplasts. Furthermore, such an activity should be specific to extracts from strains carrying the genes for B-modification.

Although no such activity could be found in crude sonicates, primarily because of interference of the extract with the infectivity assay, it was found in the dialyzed ammonium-sulfate fraction. The preparation of this active fraction is described in Materials and Methods. It is a fractionating method designed to remove primarily nonprotein material. The important steps of this purification method are removal of material precipitable at 0.035 M $MgCl_2$ and 3% streptomycin and collection of the proteins by precipitation with ammonium-sulfate (60% saturation).

Incubation of fd sB-1⁰ sB-2 RF·0 with the dialyzed ammoniumsulfate fractions prepared from strains conferring B-modification leads to a threefold higher infectivity on B-spheroplasts than that obtained after treatment of the same RF with extracts from nonmodifying strains. On the other hand, the infectivity on O-spheroplasts remains approximately the same with all enzyme preparations (Table 3). Hence the criteria mentioned above are fulfilled.

The F-prime strain 2539, which is diploid in the genes for modification and restriction, shows a 3 to 5-fold higher modification activity than normal B-strains (Table 4). This strain was therefore used as a source of extracts for further purification of the enzyme activity.

Source of extract	Infectivity on O (plaque formers per OD ₂₆₀ x 10 ⁻⁴)	Infectivity on B	Relative infectivity on B
519 = C	62	2.5,	0.04
1101 = K endo ⁻	81	2.3	0.03
707 = B r _B ⁺ m _B ⁺	47	8.1	0.18
837 = B r _B ⁻ m _B ⁺	74	7.4	0.10
834 = B r _B ⁻ m _B ⁻	78	1.3	0.02

Table 3: Effect of the dialyzed ammonium sulfate fractions from various strains of E. coli upon rd sB-1^o sB-2 RF.O. Incubations were carried out as described in Materials and Methods with 30 μ M SAM and 1 mg per ml of protein in the reaction mixture. No corrections were made for the relative competence of the spheroplast preparations.

Source of extract	Infectivity on O (plaque formers per OD ₂₆₀ × 10 ⁻⁴)	Infectivity on B (plaque formers per OD ₂₆₀ × 10 ⁻⁴)	Normalized relative infectivity on B
Omit extract	200	1.4	0.04
834 ($r_B^- m_B^-$)	150	1.6	0.05
707 ($r_B^+ m_B^+$)	30	0.6	0.10
2539 ($r_B^+ m_B^+ / r_B^+ m_B^+$)	90	5.7	0.34

Table 4: Modification activity of the dialyzed ammonium sulfate fractions from haploid and partial diploid strains. The reaction mixtures with the composition described in Materials and Methods contained 30 μ M SAM and approximately 2 mg per ml of protein. Incubations were carried out for 30 min at 30°C.

3. Properties of the reaction with the dialyzed ammoniumsulfate fraction

Experiments designed to establish the optimal conditions for the modification reaction, revealed that the dialyzed ammoniumsulfate fraction still contains some factors which interfere with the infectivity assay. As an example we will discuss in more detail the effect of the time of incubation upon the degree of modification measured as normalized infectivity on B (Figure 10).

After 30 minutes of incubation, the normalized relative infectivity on B reaches a maximum, but after further incubation it decreases again and reaches a level which is even lower than the normalized relative infectivity of untreated RF. This decrease is also observed with extracts from nonmodifying and nonrestricting strains.

If in the latter case the absolute infectivities on B-spheroplasts and on O-spheroplasts are followed, it can be seen that this decrease of the relative infectivity on B (infectivity on B divided by the infectivity on O) is caused by a decrease of the infectivity on B-spheroplasts.

The most plausible explanation for this effect is that an inhibitor of infectivity is formed during the incubation with the dialyzed ammoniumsulfate fraction. Such an inhibitor would reduce especially the yield of plaques obtained with the restricting B-spheroplasts, since in order to get a sufficient yield of plaques, ten times more reaction mixture is used in the infectivity assay than for the infectivity assay with O-spheroplasts.

The same effect was observed when the normalized infectivity on B was measured as a function of the concentration of extract in the modification reaction. If the amount of

protein added exceeded 2.0 mg per ml (incubation 30 min at 30°C), the normalized relative infectivity started to decrease again.

The nature of these factors which interfere with the infectivity was not investigated in more detail. However, because of their presence the conditions in the modification assay have to be carefully chosen and the interpretation of the normalized infectivity on B can lead to an underestimation of the real rate of modification. Optimal values of the normalized relative infectivity on B were obtained upon incubation at 30°C for 30 minutes at pH 6.7 and a protein concentration between 0.5 and 2.0 mg per ml.

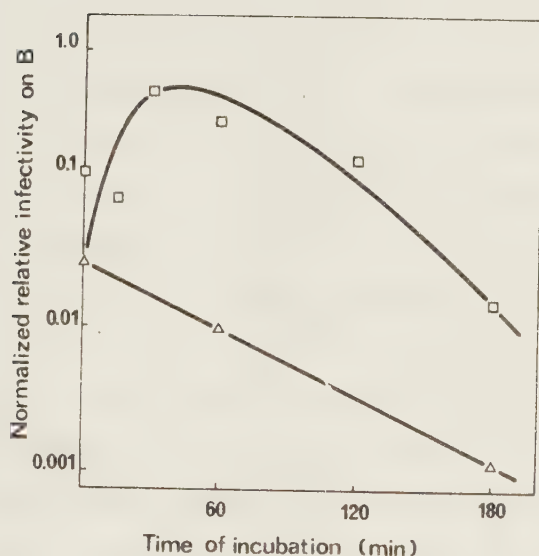


Figure 10: Apparent time dependence of the modification reaction with the dialyzed ammonium sulfate fraction. The reaction mixtures were as indicated in Materials and Methods, with 30 μ M SAM, 0.15 OD₂₆₀ units per ml of fd sB-1 sB-2 RF-0, and 2 mg per ml of protein. After incubation at 30°C for the times indicated, the degree of modification of the RF was measured by determining its normalized relative infectivity on B.

For this determination, the infectivity on B-spheroplasts was calculated from the total number of plaques obtained in two infectivity assays with 5 μ l of the reaction mixture, and the infectivity on O-spheroplasts from total number of plaques obtained in two assays with 0.5 μ l of the reaction mixture. Δ - Δ , incubation in presence of an extract from strain 834 ($r_B^- m_B^-$); $\square$ - $\square$, incubation in presence of an extract from strain 707 ($r_B^+ m_B^+$).

4. Purification of the modification activity

The dialyzed ammoniumsulfate fraction prepared from a 37-liter culture of the partial diploid strain 2539 was further purified by chromatography on phosphocellulose and DEAE-cellulose. The details of the purification procedure and the elution profiles of the columns are given in Materials and Methods.

Upon passage through the phosphocellulose column, a partial separation of the modification and restriction activity was obtained. Modification activity eluted at a mean concentration of 0.18 M potassiumphosphate, pH 6.8 and the restriction activity at a mean concentration of 0.19 M (Figure 11). Further purification of the phosphocellulose fractions containing the major modification activity on a DEAE-cellulose column gave a fraction which was free of any detectable restriction activity.

The recovery of activity and protein in the individual purification steps are summarized in Table 5. For this purpose, a unit of modification was defined as the activity which gives an increase of 0.1 to the normalized relative infectivity on B of fd sB-1⁰sB-2 RF·0 after incubation under the standard conditions described in Materials and Methods. According to the equation which correlates the normalized relative infectivity on B and the rate of modification, this increase corresponds to approximately 10% modification.

The purification by chromatography on phosphocellulose and DEAE-cellulose was approximately 150-fold, with a recovery of about 6% of the activity present in the dialyzed ammonium-sulfate fraction. The major loss occurred during the phosphocellulose step. This loss is mainly due to the instability of the enzyme in the highly diluted column fractions.

The final preparation, concentrated and stored in 50% glycerol at -15°C did not show any detectable loss of activity during several months.

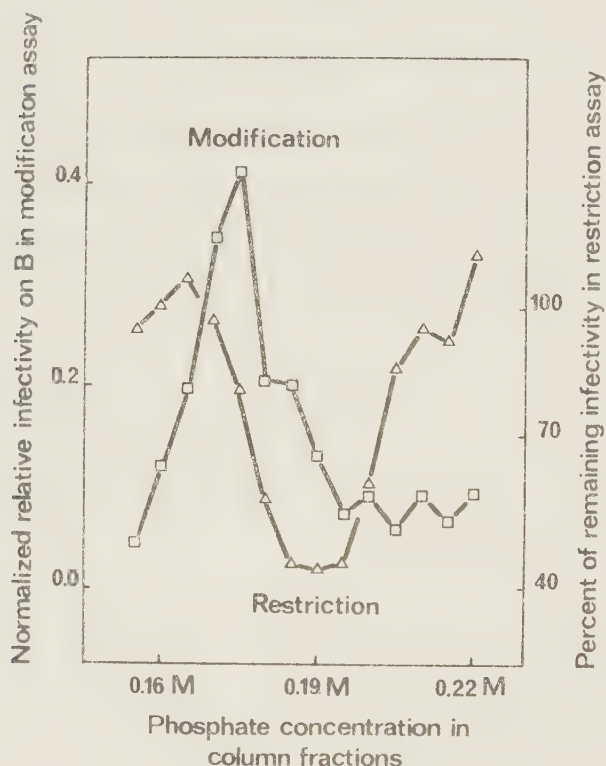


Figure 11: Partial separation of modification and restriction activities upon chromatography on phosphocellulose. The reaction mixtures (140 μl) contained 0.15 OD_{260} units per ml of fd sB-1⁰ sB-2 RF-0 and 40 μl of column eluate. The chromatography procedure is described in Materials and Methods.

□—□, normalized relative infectivity on B-spheroplasts after incubation for 30 min at 37°C in modifying conditions (pH 6.0) as described in Materials and Methods. Δ — Δ , per cent of remaining infectivity on O-spheroplasts after incubation for 30 min at 37°C in restricting conditions as described by Linn and Arber (1968).

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units per mg)
Crude extract	395	-	3320	-
Dialyzed ammonium sulfate fraction	68	2130	1490	1.43
Concentrated phospho- cellulose eluate	9.9	192	5.4	34.6
Concentrated DEAE- cellulose eluate	1.7	131	0.70	186

Table 5: Purification of the modification activity from strain 2539. Details of the purification procedure are given in Materials and Methods. No activity could be measured in the crude extract because of interference with the infectivity assay. One unit of modification is defined as the activity which gives an increase of 0.1 to the normalized relative infectivity on B of fd SB-1⁰ SB-2 RF.0 after incubation for 30 min under the conditions described in Materials and Methods.

5. Properties of the reaction with the purified enzyme fraction

After prolonged incubation of fd sB-1⁰sB-2 RF·O with the purified enzyme fraction, the RF is as infective on B-spheroplasts as is in vivo modified RF·B, indicating that virtually all of the substrate has been modified (Figure 12). In contrast to incubation with the dialyzed ammoniumsulfate fraction, no loss in biological activity is observed, even after incubation for more than 5 hours.

In potassium phosphate buffer, the reaction rate is optimal at 37°C, pH 6.0 with a Mg^{++} concentration of $7 \times 10^{-3}M$ (Figures 13 and 14). Without Mg^{++} the reaction rate is approximately 3-fold lower, but is not further inhibited by the presence of $1.4 \times 10^{-2}M$ EDTA (standard incubation mixture). Modification has an absolute requirement for SAM, but is independent of ATP. Omission of incubation of the reaction mixture gives no modification, thus indicating that the modification reaction does not take place during the infectivity assay (Table 6).

The substrate specificity of the purified enzyme fraction is shown in Table 7. As expected, the infectivity of modified fd RF·B remains unaffected by the incubation with the modification activity. Both wild-type fd RF·O (two B-restriction sites) and fd sB-1⁰sB-2 RF·O (one B-restriction site) undergo B-specific modification, the degree of which, however, differs for RF DNA from the two strains. This difference is attributed to the number of B-specific sites that must be modified on each molecule to ensure full infectivity on B-spheroplasts. Finally, the relative infectivity on B of unmodified, single-stranded fd DNA is not measurably affected by incubation with the modification enzyme.

The rate of modification of fd sB-1⁰ sB-2 RF·O, measured as the increase of the normalized relative infectivity on B, is proportional to the amount of enzyme added (Figure 15). It seems, therefore, that the modification enzyme has to catalyze a single reaction per RF molecule in order to protect it against restriction by the B-spheroplasts.

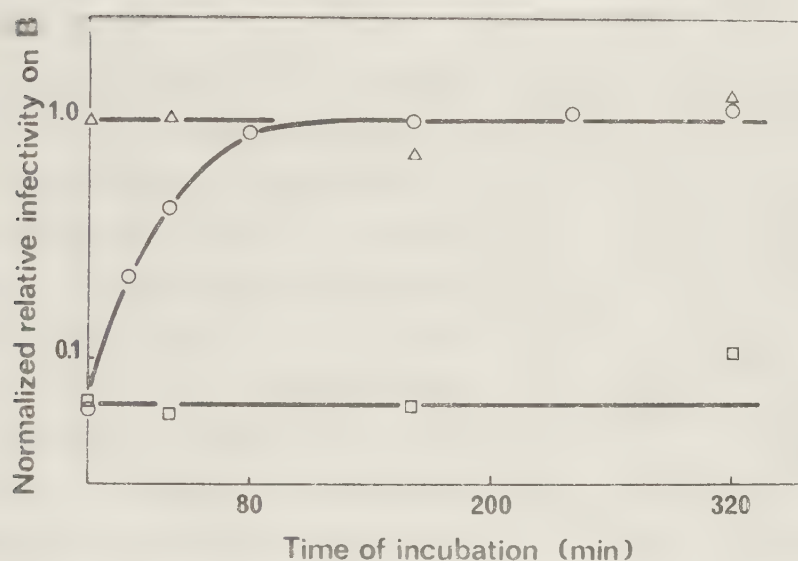


Figure 12: Time dependance of the modification reaction with the DEAE-cellulose eluate. The reaction mixtures with the compositions described in Materials and Methods (300 μ l, pH 6.0) contained 0.25 OD₂₆₀ units per ml of RF and 8×10^{-2} mg per ml of purified enzyme and were incubated at 57°C. At the times indicated, samples of 20 μ l were removed and the normalized relative infectivity determined. o, fd sB-1⁰ sB-2 RF·O, complete system; Δ, fd sB-1 sB-2 RF·B, complete system; □, fd sB-1⁰ sB-2 RF·O, omit enzyme.

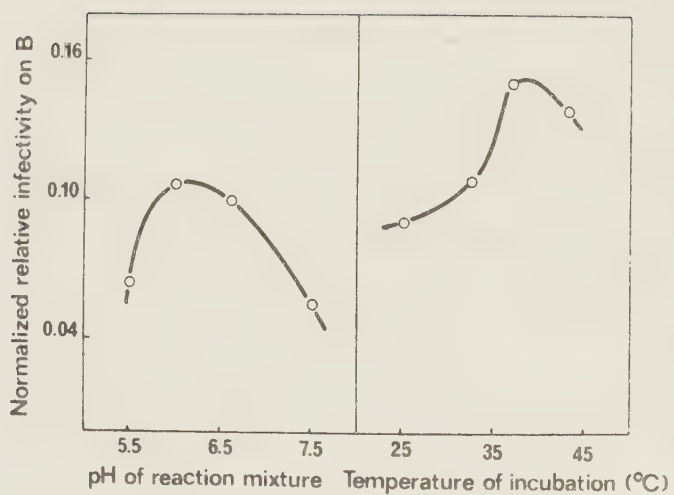


Figure 13: Temperature and pH dependence of the rate of modification of fd sB-1⁰sB-2 RF·0. The reaction mixtures with the composition described in Materials and Methods contained 40 μ M SAM and 5×10^{-5} mg per ml of purified enzyme. In the case of the temperature dependence, the reaction was carried out at pH 6.0, and in the case of the pH dependence the incubation was carried out at 30°C. The time of incubation was 40 min.

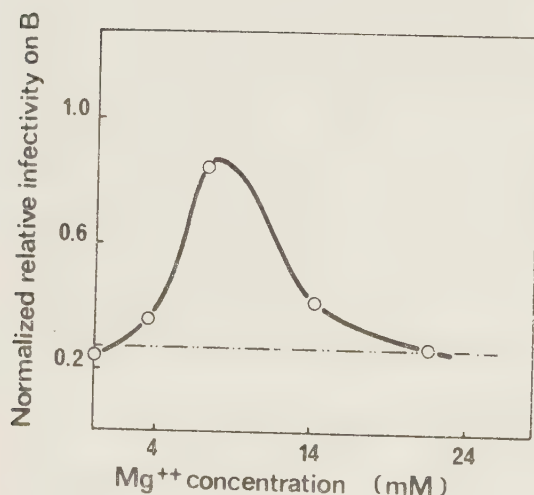


Figure 14: The dependence of the modification reaction upon the Mg^{++} concentration. fd sB-1⁰sB-2 RF·0 was exposed to 5×10^{-5} mg per ml of purified enzyme as indicated in Materials and Methods, except that EDTA was omitted, and $MgCl_2$ was added at the concentrations indicated. Incubation was for 40 min at pH 6.0. The base line (---) indicates the normalized relative infectivity on B obtained after incubation with 2×10^{-2} M EDTA and no $MgCl_2$ in the reaction mixture.

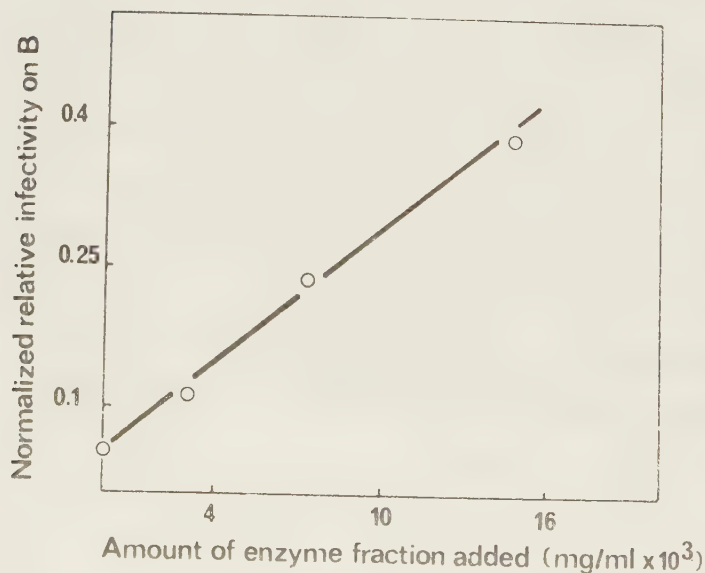


Figure 15: Dependence of the rate of modification of fd sB-1⁰sB-2 RF 0 upon the enzyme concentration. The reactions were carried out as indicated in Materials and Methods, with an incubation time of 30 min.

Reaction mixture	Infectivity on O (plaque formers per OD ₂₆₀ x 10 ⁻⁴)	Infectivity on B	Normalized relative infectivity on B
Omit enzyme	180	12	0.03
Complete system	230	58	0.10
Omit EDTA, add 7 mM Mg ⁺⁺	240	140	0.24
Omit EDTA, add 7 mM Mg ⁺⁺ and 1.4 mM ATP	150	110	0.30
Omit 2-mercaptoethanol	230	61	0.11
Omit S-adenosylmethionine	210	24	0.05
Complete, but enzyme heated 5 min at 100°C	170	22	0.05
Omit 37°C incubation	190	20	0.04

Table 6: Conditions for in vitro modification of fd sB-1 sB-2 RF·O. The RF was exposed to 3×10^{-3} mg per ml of the purified enzyme fraction as described in Materials and Methods with the alterations specified. Incubations were carried out for 30 min at 37°C and at pH 6.0.

Substrate	Reaction mixture	Infectivity on O (plaque formers per OD ₂₆₀ x 10 ⁻⁴)	Infectivity on B (plaque formers per OD ₂₆₀ x 10 ⁻⁴)	Normalized relative infectivity on B
fd <u>SB-1</u> <u>SB-2</u> RF·B	Omit enzyme Complete system	34 28	490 370	= 1 0.92
fd <u>SB-1</u> <u>SB-2</u> RF·O	Omit enzyme Complete system	34 10	0.95 26	0.002 0.18
fd <u>SB-1</u> ⁰ <u>SB-2</u> RF·O	Omit enzyme Complete system	15 22	9.1 250	0.04 0.79
single-stranded fd <u>SB-1</u> <u>SB-2</u> ·O DNA	Omit enzyme Complete system	18 6.1	0.61 0.24	0.002 0.003

Table 7: Substrate specificity of the modification activity. Assays were as described in Materials and Methods, except that the reaction mixtures contained 3×10^{-3} mg per ml of the purified enzyme fraction, 7 mM Mg^{++} , and no EDTA and were at pH 6.0. Incubations were carried out for two hours at 37°C .

6. In vitro restriction of in vitro modified RF

In a further experiment, fd sB-1⁰sB-2 RF·O was partially modified in vitro and used as a substrate in a subsequent restriction assay. The normalized relative infectivity on B after exposure to the modification enzyme was 0.33, indicating that 30% of the molecules had been modified (Table 8). This partially modified RF-sample was threefold more resistant to restriction by endonuclease R·B than was untreated RF (Table 8, infectivity on O). This difference in inactivation is somewhat less than the expected 5-fold difference. However, it has been shown that the restricting enzyme fraction (purified by chromatography on phosphocellulose) shows still some unspecific inactivation of fd RF B (S. Linn, Personal

ommunication). The last column of Table 8 shows the normalized relative infectivity on B of the RF-samples after treatment with the restriction enzyme. The normalized relative infectivity on B of the unmodified RF remains unchanged, indicating that the restricting enzyme fraction contained no modification activity. On the other hand, the normalized relative infectivity on B of the previously modified RF-sample shows a further increase upon treatment with the restriction enzyme. This increase is attributed to selective inactivation of the unmodified RF molecules still present in the partially modified sample.

In contrast to the inactivation by the endonuclease R·B, the inactivation of RF by endonuclease R·P1 is not affected by B-modification of the RF. A sample of fd sB-1⁰sB-2 RF·O was modified in vitro and its restriction by the dialyzed ammonium sulfate fraction prepared from the P1-lysogenic strain 930, was compared with the restriction of in vivo B-modified and unmodified RF. This experiment is summarized in Table 9. In the modification reaction 70% of the molecules present were modified (Table 9, part A). The inactivation of

this partially modified RF sample upon incubation with the dialyzed ammoniumsulfate fraction containing endonuclease R·P1 is approximately 7-fold if SAM is omitted in the reaction and approximately 100-fold for the complete reaction mixture. (Table 9, part B). This stimulation of inactivation by the addition of the cofactor of the restriction enzyme, SAM, is attributed to the presence of the P1-restriction enzyme. The same values of inactivation are found for in vivo modified RF and unmodified RF. Thus, the B-modification does not influence the action of the P1-restriction enzyme and the high degree of specificity displayed by in vivo modification is preserved in vitro.

In vitro modification		In vitro restriction assay			
Conditions	Normalized relative infectivity on B after 40 minutes incubation	Assay conditions	Infectivity on O (plaque formers per $OD_{260} \times 10^{-4}$)	Infectivity on B	Normalized relative infectivity on B
omit modification enzyme	0.04	omit restriction enzyme	52	1.0	0.03
		phosphocellulose restriction fraction added	3.3	0.1	0.04
DEAE-cellulose modification fraction added	0.33	omit restriction enzyme	65	15	0.32
		phosphocellulose restriction fraction added	12	6.7	0.78

Table 8: Restriction in vitro of fd sB-1⁰sB-2 RF-0 modified in vitro. The reaction mixtures for modification (155 μ l) contained 0.4 OD_{260} RF per ml and, where indicated, 2×10^{-2} mg per ml of purified enzyme. Incubation was carried out for 40 min at 37°C in potassium buffer pH 5.0. The mixtures were assayed for infectivity, then dialyzed overnight versus 10^{-4} M EDTA, 0.01 M Tris-HCl (pH 8.5). Fifty μ l of the dialyzed material was used as a substrate in a subsequent restriction assay. The B-specific restriction enzyme had been purified on phosphocellulose and was free of modification activity. Conditions for the restriction assay were the same as described by Linn and Arber (1968).

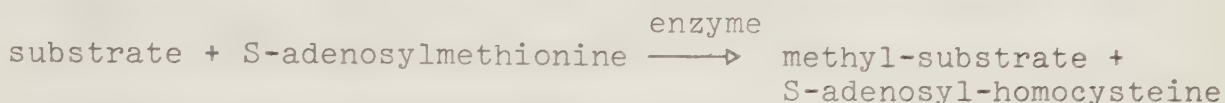
<u>In vitro</u> B-specific modification (A)			<u>In vitro</u> P1-specific restriction (B)	
Substrate	Conditions	Normalized relative infectivity on B	Assay conditions	Remaining infectivity on O-spheroplasts (%)
fd <u>SB-1</u> <u>SB-2</u> ⁰ RF·O	Complete system	0.74	Omit enzyme Omit SAM Complete system	= 100 14.5 0.5
	Omit enzyme	0.04	Omit enzyme Omit SAM Complete system	= 100 14.3 1.2
	Omit enzyme	= 1	Omit enzyme Omit SAM Complete system	= 100 23.5 1.9

Table 9: Influence of B-modification of RF on its restriction by the dialyzed ammonium sulfate fraction of the P1-lysogenic strain 930. The reaction mixtures for modification, with the compositions described in Materials and Methods, contained 0.3 OD₂₆₀ units of RF per ml and, where indicated, 4 x 10⁻² mg per ml of purified enzyme. After one hour of incubation at 37°C, the mixtures were dialyzed overnight versus 10⁻⁴ M EDTA, 0.01 M Tris-HCl (pH 8.5). Fifty µl of the dialyzed material was exposed to 4 mg protein per ml of the dialyzed ammonium sulfate fraction of the P1-lysogenic strain 930 in restricting conditions as described by Linn and Arber (1968). Incubations were carried out for 20 min at 37°C.

IV. THE CHEMICAL NATURE OF MODIFICATION

1. Correlation of modification and methylation of DNA

S-adenosylmethionine (SAM), the cofactor of the modification enzyme, is known as the donor of methyl groups in many enzyme catalyzed methylation reactions. In such reactions a methyl group is transferred from the S-adenosylmethionine according to the following equation :



The absolute requirement of SAM for in vitro modification suggests that the modification enzyme is a methylase.

This was demonstrated in an experiment described in Figure 16. A sample of fd RF.O was modified in vitro in presence of SAM which was tritium labelled in the methylgroup. After the incubation of the reaction mixture, the SAM was removed by dialysis and the sample was analyzed on a sucrose gradient.

The infectivity profile shows two peaks. The ratio of their sedimentation constants corresponds to the ratio expected for the two infective forms of RF, RF I (24 s, supercoiled) and RF II (19 s, one strand nicked). The profile of H^3 -counts coincides with the infectivity profile, indicating that both, RF I and RF II had been methylated.

In a control experiment, the in vivo modified replicative form, fd RF.B, was used as a substrate in the modification reaction. As expected, no significant incorporation of H^3 -counts could be observed, since the modification sites are already occupied by unlabelled methyl groups.

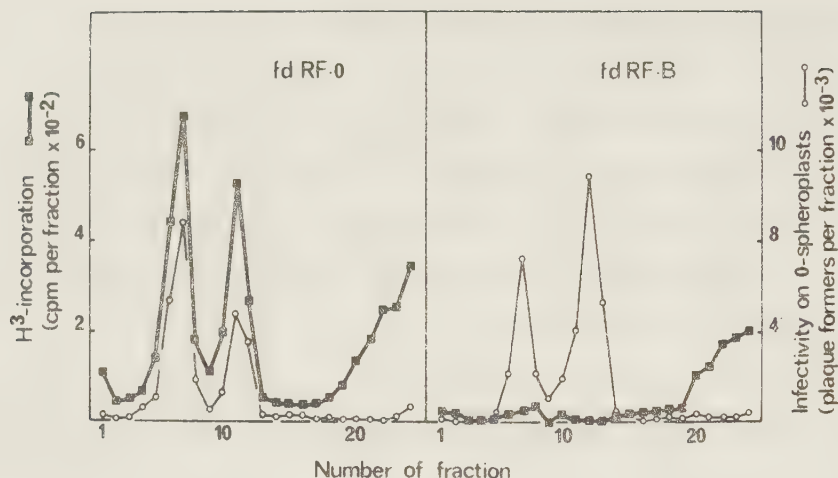


Figure 16: Incorporation of methyl groups into fd SB-1 SB-2 RF. Samples of fd RF-0 and fd RF-B were exposed to the modification enzyme in presence of H³-methyl S-adenosylmethionine (SAM-H³) and analyzed on a sucrose gradient. The reaction mixtures (130 μ l) with the composition described in Materials and Methods contained 50 μ M SAM-H³ (specific activity 4.2 C per mM), 5×10^{-2} mg per ml of the purified enzyme fraction and 2.5×10^{-2} mg per ml of RF. After incubation for 5 hours at 37°C the reaction mixtures were dialyzed for 60 hours against 4 x 500 ml of 10^{-3} M EDTA, 0.02 M glycine-NaOH (pH 9.5). The two samples were layered on 5 ml sucrose gradients (5%-20% sucrose in 10^{-3} M EDTA, 0.02 M glycine-NaOH; pH 9.5). After centrifugation in a SW 39 Spinco rotor for 6 hours at 35,000 rpm at 15°C, a hole was punched in the bottom of the tube and fractions of 200 μ l were collected. The H³-counts of the fractions (■-■) were determined by counting samples of 100 μ l in 10 ml Bray-scintillation liquid in a scintillation counter. The infectivity (o-o) was determined by plating on 99l-spheroplasts.

2. The relative incorporation of methyl groups into the RF of restriction deficient mutants of phage fd

As pointed out previously, the restriction enzyme and the modification enzyme of a given host-specificity system act on the same sites on the substrate DNA. Such sites are presumably determined by their base sequence. Recent investigations of the genetic determinants of host-specificity indicate, that the two enzymes carry a common subunit which is responsible for the recognition of such sites (Boyer and Roulland-Dussoix, 1969).

In consequence of this model, a mutation of a specificity site which prevents its recognition by the restriction enzyme should result simultaneously in the loss of its accessibility to the modification enzyme. The single-step mutant of phage fd, fd sB-1⁰sB-2, which has a reduced sensitivity towards B-restriction, and the double mutant fd sB-1⁰sB-2⁰ which shows no B-restriction at all, have been interpreted to have lost one and both restriction sites, respectively. Thus, if the above model is right, the methyl groups which can be transferred by the B-modification enzyme to the RF of the mutant fd sB-1⁰sB-2 should be half the number of methyl groups which can be transferred to the RF of the wild type phage. The double mutant fd sB-1⁰sB-2⁰ should have lost all the receptor sites for methyl groups.

The results obtained agree with the expected values. Different amounts of RF were modified in vitro under conditions which lead to the completion of the methylation reaction (Table 10). The amount of H³ incorporated per RF molecule of fd sB-1⁰sB-2 was 49% of the amount of H³ incorporated per RF molecule of the wild type phage, whereas in the double mutant fd sB-1⁰sB-2⁰ the incorporation was reduced to less than 2%. The modified wild type RF, fd RF·B, still shows a measurable amount of H³ incorporation (7% of the unmodified wild type). This residual incorporation might be attributed to incomplete in vivo modification.

In order to quantitate the incorporation of H^3 -label into the RF DNA, the RF samples to be tested were mixed with P^{32} -labelled fd sB-1⁰sB-2⁰ RF·B. The SAM- H^3 which had not undergone reaction was separated from the RF by filtration on Sephadex G 100. The RF contained in the column fractions was precipitated, collected on filters and the ratio P^{32}/H^3 was determined. The H^3 -backgrounds obtained were in the order of 20 cpm to 50 cpm. The method to determine this background, the details of the purification method and the preparation of P^{32} -labelled RF are described in Materials and Methods.

The absolute number of methyl groups incorporated per RF molecule can be calculated from the specific activity of the tritiated SAM. However, this value given by the producing firm is probably not very reliable and the numbers obtained can therefore only be considered as a rough estimate. The values obtained are 3.2 methyl groups per RF molecule for the mutant fd sB-1⁰sB-2 and 6.5 methyl groups per RF molecule for the wild type phage.

Substrate	RF concentration of reaction mixture (mg per ml x 10 ²)	Incorporation of methyl groups		
		Ratio H ³ -counts/P ³² -counts	H ³ -counts per mg of RF (cpm x 10 ⁻³)	Relative incorporation of methyl groups per RF molecule
fd <u>SB-1</u> <u>SB-2</u> RF·O	0.73	0.72	4320	= 100%
	1.45	1.35	4060	
fd <u>SB-1</u> ⁰ <u>SB-2</u> RF·O	0.75	0.36	2100	49%
	1.39	0.65	2100	
	1.50	0.67	1950	
fd <u>SB-1</u> ⁰ <u>SB-2</u> ⁰ RF·O	1.45	0.03	90	2%
fd <u>SB-1</u> <u>SB-2</u> RF·B	1.58	0.10	280	7%

Table 10: Relative incorporation of methyl groups into different types of RF. The reactions were carried out as indicated in Materials and Methods. The volume of the reaction mixtures was 50 μ l and contained 80 μ M of SAM-H³ (specific activity 4.2 C per mM), 6 x 10⁻² mg per ml of the purified enzyme fraction, 43,600 cpm per ml of P³²-labelled fd SB-1⁰ SB-2⁰ RF·B (approximately 4 x 10⁻⁴ mg per ml), and the amount of unlabelled RF indicated. Incubation was carried out for 12 hours at 57°C. The SAM-H³ which had not undergone reaction was removed and the incorporation of H³ was determined as indicated in Materials and Methods.

3. Kinetics of the incorporation of methyl groups

The incorporation of methyl groups into fd sB-1⁰sB-2 RF.0 as a function of the time of incubation is represented in Figure 17. After 180 min of incubation, the incorporation of methyl groups, measured as ratio H^3 -counts/ P^{32} -counts, reaches a saturation level. Upon further addition of RF, the incorporation of methyl groups resumes, indicating that the plateau is not due to exhaustion of SAM or inactivation of the enzyme during the reaction.

From the results obtained in this experiment, the values $1/E \times \ln (M_0/M_0 - M)$ were calculated. In this formula E is the concentration of enzyme, M_0 the saturation level of methyl incorporation and M the level of methylation at time t . In Figure 18, the calculated values are plotted versus the time of incubation. A straight line is obtained which passes through the origin of the graph. This is the result expected of a first order reaction. In this case, the slope of the straight line equals the velocity constant of the reaction.

The same experiment was carried out with the RF of the wild type phage instead of the mutant fd sB-1⁰sB-2 as substrate. Again, the reaction follows first order kinetics and the velocity constant is the same as for the mutant RF (Figure 18). As we have shown previously, the wild type RF carries two specificity sites on its DNA. The above observation indicates that those two sites are equivalent.

In agreement with first order kinetics, the velocity of the incorporation of methyl groups is proportional to the enzyme concentration (Figure 19).

The dependence of the reaction velocity on the SAM concentration is represented in Figure 20. Half of the maximal velocity is reached at a concentration of approximately 4×10^{-3} mM SAM. This value agrees with the Michaelis constant obtained from the Lineweaver-Burk plot (Figure 21). The

experimental value and the theoretical value for the maximal reaction velocity are the same, indicating that excess SAM does not inhibit the reaction.

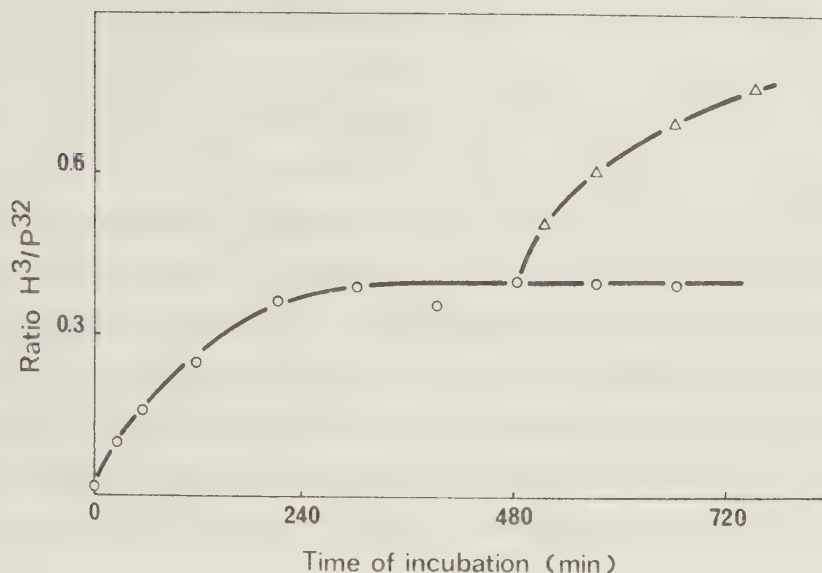


Figure 17: Incorporation of methyl groups in function of the time of incubation. The reaction mixture (400 μ l) with the composition described in Materials and Methods contained 90 μ M SAM-H³ (specific activity 4.2 C per mM), 5.2×10^{-2} mg per ml of the purified enzyme fraction, 54,600 cpm per ml of P³²-labelled fd sB-1^osB-2^oRF·B (approximately 3.3×10^{-5} mg per ml) and 1.1×10^{-2} mg per ml of fd sB-1^osB-2 RF·O. At the times indicated, aliquots of 20 μ l were removed and the ratio H³-counts/P³²-counts of the RF was determined as described in Materials and Methods (o). After 480 min of incubation, further fd sB-1^osB-2 RF·O was added to a fraction of the reaction mixture to give a final RF concentration of 2.0×10^{-2} mg per ml and the incubation was continued (Δ).

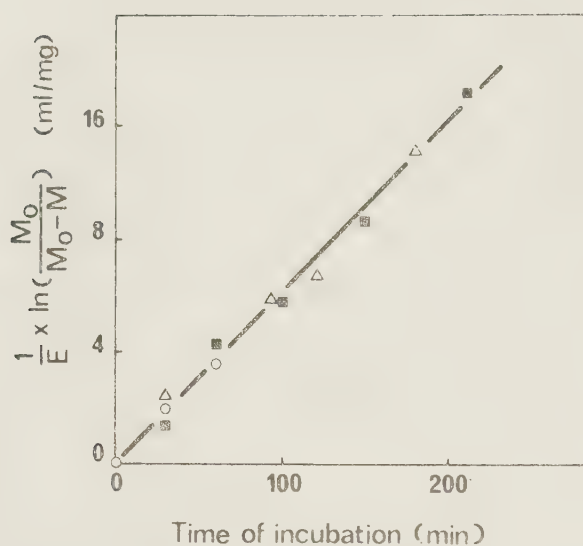


Figure 18: Determination of the order of the methylation reaction. From the data represented in Figure 17, the values

$$\frac{1}{E} \times \ln\left(\frac{M_0}{M_0 - M}\right)$$

were calculated and plotted versus the time of incubation. E = enzyme concentration (mg per ml), M_0 = saturation value of the ratio H^3 -counts/ P^{32} -counts, M = ratio H^3 -counts/ P^{32} -counts at time t. The

points were calculated from the beginning of the reaction ($M_0 = 0.4$; o) and from the reaction where additional RF had been added after 480 min of incubation ($M_0 = 0.8$; Δ). A similar experiment was carried out with fd sB-1 sB-2 RF·0 as substrate (■). In this reaction the SAM- H^3 concentration was $54 \mu M$ and the enzyme concentration 4.7×10^{-2} mg per ml.

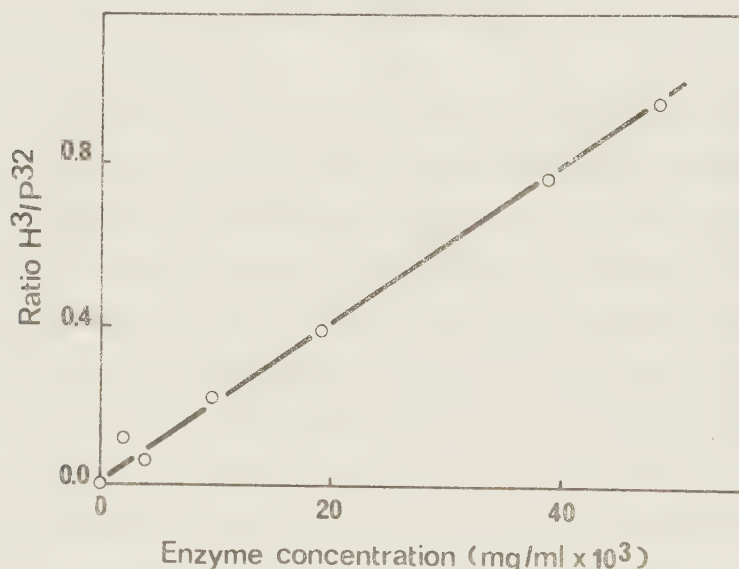


Figure 19: Dependence of the reaction velocity upon the enzyme concentration. The reaction mixtures (105 μl) contained $35 \mu M$ SAM- H^3 (specific activity 4.7 C per mM), 3.6×10^{-2} mg per ml of fd sB-1 sB-2 RF·0 and the amounts of enzyme indicated. The volumes of the reaction mixtures were adjusted with 50% glycerol. After incubation for 40 min at $57^\circ C$, 820 cpm of P^{32} -labelled fd sB-1 sB-2 RF·B were added and the ratio H^3 -counts/ P^{32} -counts was determined as described in Materials and Methods.

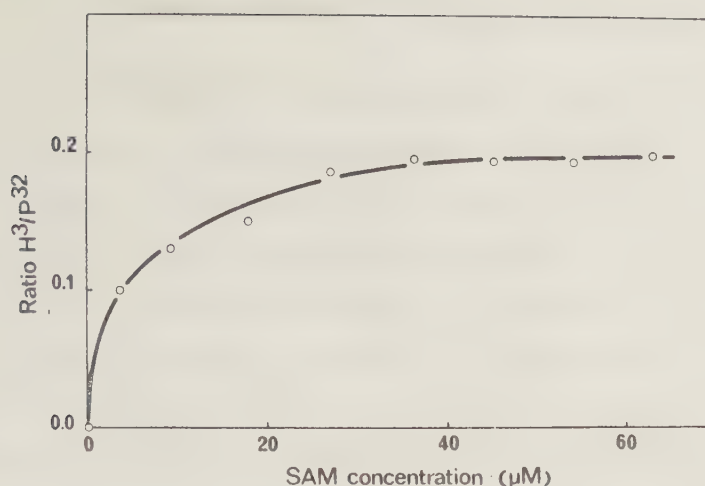


Figure 20: Dependence of the reaction velocity upon the concentration of SAM. The reaction mixtures (100 μ l) contained 1.4×10^{-2} mg per ml of the purified enzyme fraction, 5.1×10^{-2} mg per ml of fd sB-1^osB-2 RF·O and the amount of SAM-H³ (specific activity 4.7 C per mM) indicated. After incubation for 40 min at 57°C, 1290 cpm of P³²-labelled fd sB-1^osB-2^o RF·B were added to each reaction mixture and the ratio H³-counts/P³²-counts was determined as indicated in Materials and Methods.

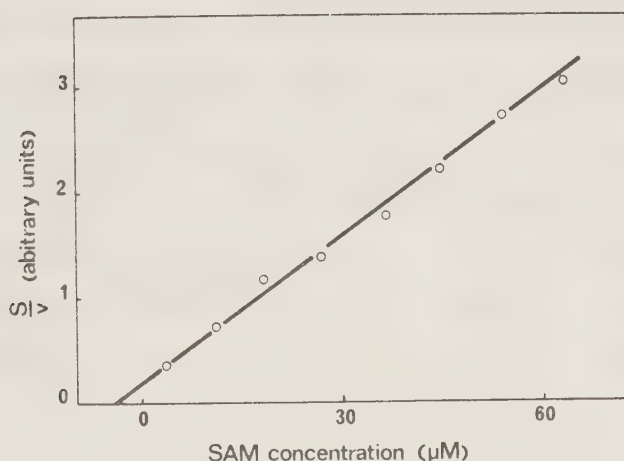


Figure 21: Lineweaver-Burk of the SAM-dependence of the velocity of the methylation reaction. From the results represented in Figure 20, the values S/v (S = concentration of SAM, v = ratio H³-counts/P³²-counts after 40 min of incubation) were plotted versus the SAM concentration. A straight line is obtained, indicating that the Michaelis-Menten equation is fulfilled. In this case, the intercept with the abscissa corresponds to the negative value of the Michaelis constant and the slope of the straight line is equal to the reciprocal value of the maximal reaction velocity.

4. The chemical nature of the modified bases

The only methylated bases known to occur in the DNA of microorganisms, as well as higher organisms, are a methylated derivative of cytosine, 5-methylcytosine, and a methylated derivative of adenine, 6-methylaminopurine (see Srinivasan and Borek, 1966). In order to ensure that modification involves covalent attachment of methyl groups to some of the bases of the substrate DNA, and to decide between the two possible candidates, adenine or cytosine, we have analyzed the bases of modified DNA by chromatography.

The replicative form DNA of fd was modified in vitro with tritiated S-adenosylmethionine as the donor of the methyl groups. The SAM-H^3 was removed and the fd RF hydrolyzed with formic acid. This hydrolysis procedure liberates the bases of the DNA. They were analyzed by chromatography on paper together with markers of 6-methylaminopurine and 5-methylcytosine. All the radioactivity is found to migrate together with 6-methylaminopurine (Figure 22).

A similar experiment was carried out with DNA isolated from phage λ grown on the O-strain 921 ($r_K^- m_K^-$; Wood, 1966) and bacterial DNA isolated from the $r_K^+ m_K^+$ strain 991. In both cases the radioactivity was found associated with 6-methylaminopurine, indicating that B-modification is generally conferred to DNA by a specific methylation of adenine (Figure 23).

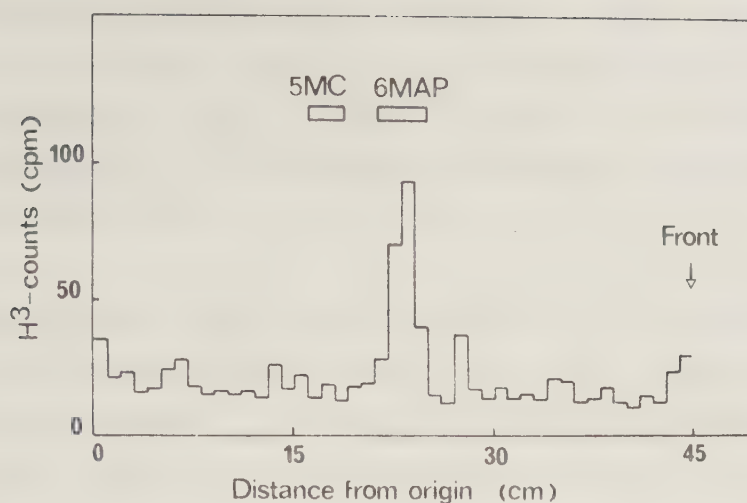


Figure 22: Chromatogram of the methylated bases of in vitro modified fd sB-1 sB-2 RF 0. The reaction mixture for modification was as indicated in Materials and Methods with 5.3×10^{-3} mg per ml of the purified enzyme fraction, 5×10^{-2} mg per ml of fd RF 0 and $14 \mu\text{M}$ SAM- H^3 (specific activity 4.7 C per mM). Incubation was overnight at 37°C . The RF was purified from SAM- H^3 , hydrolyzed and the bases separated by chromatography on paper as indicated in Materials and Methods. The squares indicate the region of authentic 5-methylcytosine and 6-methylaminopurine localized by their absorption of ultraviolet light.

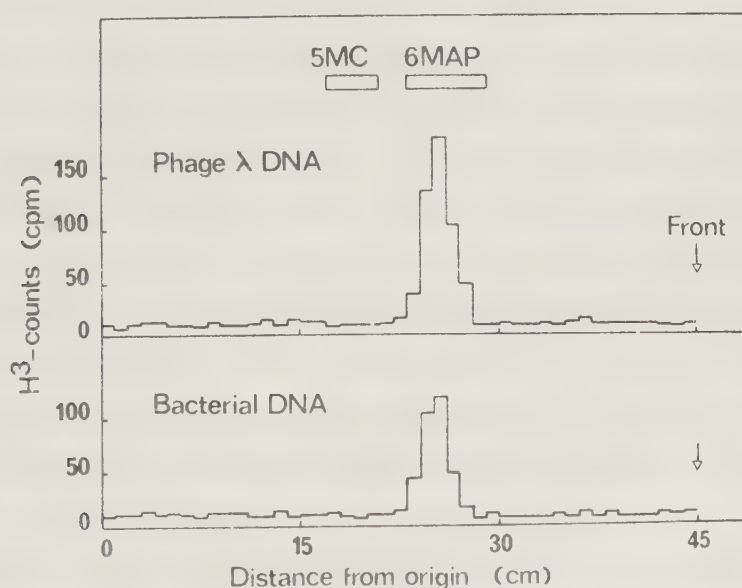


Figure 23: Chromatogram of the methylated bases of in vitro B-modified phage λ DNA and bacterial DNA. The λ -DNA had been extracted from phage grown on the O-strain 921 ($r_K^- m_K^-$; Wood, 1966) and the bacterial DNA from the $r_K^+ m_K^+$ -strain 991. The conditions for modification and chromatography were the same as in Figure 22.

V. DISCUSSION

The enzymatic activity described here was related to in vivo B-specific modification by an assay based on the biological consequences of modification. The in vitro activity renders unmodified fd RF.O resistant to B-restriction in vivo and in vitro, but it does not affect the infectivity of fd RF.B. Furthermore, the activity is specific to B strains and is not found in K or C strains. A mutation causing a restriction- and modification-less phenotype ($r_B^- m_B^-$) also results in the loss of the in vitro activity, whereas a mutant with restriction-deficient but normal modification phenotype ($r_B^- m_B^-$) shows full modification.

In potassium phosphate buffer, the reaction rate is maximal at pH 6.0. Despite the low pH optimum, which reflects unphysiological assay conditions, the high degree of specificity displayed by in vivo modification seems to be preserved in the in vitro reaction. In vitro B-modified fd RF is protected against endonucleolytic attack by the B-restriction enzyme, but is still fully accessible to the attack by the heterospecific P1-restriction enzyme.

The chemical nature of B-modification is now firmly established to be a methylation of certain bases of the DNA. The methylated bases were identified as 6-methylaminopurines. This was the result expected, since the B-strain used to isolate the modification activity is known to contain exclusively 6-methylaminopurines and no 5-methylcytosine in its DNA (Gough and Lederberg, 1966). However, the modification methylase seems to be responsible for only a minor fraction of the methylated bases. Mutants of E.coli B which are deficient in their modification phenotype still contain approximately the same number of 6-methylaminopurines in their DNA as the wild type strain. Similarly, no difference in the content of 6-methylaminopurines of the DNA of phage λ grown on the modification-deficient strain or the wild type strain could be found (Smith, Wood and Arber; cited in Arber, 1965 b).

The biological function of these major DNA-methylases, as well as the RNA-methylases, is unknown. They are found in many organisms, including bacteriophage, and also in the cells of higher organisms. They are species specific and provide the nucleic acids with well defined methylation patterns (see Borek and Srinivasan, 1966).

Recently, Yuan and Meselson (1970), in their study of the mechanism of K-restriction, discovered that the endonucleolytic cleavage is preceded by a complex formation between DNA and enzyme. This complex is not formed with modified DNA, indicating that modification does not protect DNA by preventing directly the catalytic action of the restriction enzyme, but by preventing the recognition of K-specificity sites on the substrate DNA. This mechanism provides a new potentiality to methyl groups : they are capable of influencing processes where protein-base sequence recognition is involved.

The structural relationship between the modification and restriction enzymes is not yet established. Purification of extracts from E.coli $r_B^+ m_B^+$ strains leads to a separation of the two activities. However, the two enzymes are not independent of each other. Complementation experiments (Boyer and Roulland-Dussoix, 1969) indicate that at least three gene products are involved in host-specificity. These results and the genetic investigations of Hubak and Glover (1970), are compatible with the model that two of the three gene products are necessary for the formation of the modification enzyme, whereas all three gene products are necessary for the formation of the restriction enzyme. One of the subunits common to the two enzymes seems to be responsible for the recognition of the specificity sites.

A close structural relationship between modification and restriction enzymes is supported by the following observations. First, the restriction enzyme requires S-adenosylmethionine for complex formation with the substrate DNA (Yuan and Meselson, 1970).

However, the substrate DNA is not methylated during the restriction reaction. The requirement for S-adenosylmethionine might therefore reflect that the modification enzyme or one of its subunits is a structural part of the restriction enzyme. Secondly, the mutants of phage fd which have lost their sensitivity towards restriction have lost their capacity to be modified. Considering the different reactions carried out by the two host-specificity enzymes, cleavage of the DNA for the restriction enzyme, and methylation for the modification enzyme, the mutations in the specificity sites probably do not directly influence the chemical reactions catalyzed by the two enzymes. It is more likely that they interfere with the binding of the enzymes to the DNA. Such mutations would then be similar to the mutations in phage λ which cause the virulent phenotype by preventing the binding of the λ -repressor to the phage DNA (Ptashne and Hopkins, 1968), or to the operator mutations of the lac-operon of E.coli which prevent the recognition of the operator region on the DNA by the lac-repressor (W. Gilbert, personal communication). This interpretation would be in agreement with the model which proposes a recognition subunit which is common to the restriction and the modification enzyme.

Since the restriction and the modification enzyme of the B-specificity system have now been isolated, the proposed relationship between modification and restriction enzyme is accessible to direct chemical analysis.

The biological significance of host-specific modification and restriction is not yet fully explored. In E.coli and several other microorganisms it might limit the exchange of genetic material to strains of the same species. In addition, it could represent a defense mechanism against an invasion of viruses from a different ecological environment. In higher organisms, such control mechanisms have not been explored.

The main reason that the study of the molecular mechanism of host-specificity systems is attractive, is that it involves

sequence recognition by proteins. Such processes play an important role in the control of gene transcription and possibly also in cell differentiation. The increasing number of host-specificity systems, each recognizing its own specific base sequence, together with the apparent similar structure of the enzymes involved, should allow us to explore the basic principles of the mechanism of sequence recognition. A comparison of the base sequences of the sites recognized by the different host-specificity enzymes should give some insight into the general structure of such sites (loop formation, unusual base pairing). Furthermore, the bases within a site which determine its specificity towards a particular host-specificity system could be identified. In addition, the determination of the chemical and physical properties of the corresponding recognition sub-units, especially the comparison of peptide fragments, might allow us to identify those amino acid sequences which are directly involved in sequence recognition.

For this larger project the isolation and purification of the different modification and restriction enzymes is necessary. The modification enzymes permit the labelling of the specificity sites of the DNA with radioactive methyl groups, thereby facilitating the determination of the base sequence.

The work presented here describes the isolation and characterization of the B-modification activity. Knowledge of the behaviour of the B-modification enzyme during chromatography and the basic properties of the modification reaction should now allow us to isolate the modification enzymes of other host-specificity systems.

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